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(54) Title: CHROMOGENIC 7- OR 8-POSITION MODIFIED N-ACETYLNEURAMINIC ACID SUBSTRATES AND METHODS FOR DIAGNOSING HUMAN INFLUENZA THEREWITH

(57) Abstract

Chromogenic derivatives of N-acetylneuraminic acid modified in the 7- or 8-position are used as substrates in colorimetric assays for human influenza neuraminidase activity in clinical specimens for the purpose of selectively diagnosing influenza infection. The substrates may exhibit different reactivity with the different types of influenza neuraminidases, thus enabling one to discern the specific type of influenza infection and prescribe appropriate treatment and/or supportive therapy therefor.

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CHROMOGENIC 7- OR 8-POSITION MODIFIED N-ACETYLNEURAMINIC ACID SUBSTRATES AND METHODS FOR DIAGNOSING HUMAN INFLUENZA THEREWITH

Technical Field

The present invention relates to reagents and assays for diagnosing human influenza. More specifically it relates to novel chromogenic 7- or 8-position modified N-acetylneuraminic acid substrates that are useful in the diagnosis of influenza through the detection of the enzymatic activity of human influenza neuraminidase (NA).

Background of the Invention

Influenza virus averages 30-50 million infections annually in the United States alone. Epidemiologic studies of influenza epidemics estimate the incidence of infection to be 25% in the general population and higher in school age children. Researchers have estimated that up to half the infected persons would see a physician because of the illness. In 1986, the Center for Disease Control (CDC) estimated that influenza epidemics have been associated with 10,000 or more excess deaths in 18 of the preceding 28 years. CDC studies indicate influenza as the fifth leading cause of death in the United States. Antigenic variations in the surface glycoproteins of influenza A and B account for their continued epidemics.

Influenza viruses possess surface glycoproteins that have NA activity. These glycoproteins are members of a family of neuraminidases that are found in viruses, bacteria, mycoplasmas, and animal tissues. They

hydrolyze substrates that contain alpha-ketosidically linked N-acetylneuraminic acid (Neu5Ac; referred to previously as "NANA"). In viruses, NA typically constitutes 5-10% of the viral protein and exists as a mushroom-shaped spike on the envelope. Viral NA is composed of a hydrophilic area which includes the catalytic site of the enzyme and a hydrophobic area that is inserted into the viral envelope anchoring the enzyme to the virus.

Various assays for NA activity are described in the literature. Santer, U.V., et al., Biochimica et Biophysica Acta 523:435-442 (1978), describes a colorimetric assay for NA using 2-(3-methoxyphenyl)-N-acetyl-alpha-D neuraminic acid as a substrate and 4-aminoantipyrine in the presence of an oxidizing agent to measure the enzymatically released methoxyphenol. Myers, R.W., et al., Analytical Biochemistry 101:166-174 (1980), describes the use of the 4-methylumbelliferyl-alpha-ketoside of Neu5Ac in a fluorometric assay for NA. This chromogenic derivative of Neu5Ac was also used in studies of the NA activity of influenza viruses by Yolken, R.H., et al., J. Infectious Diseases 142:5116-523 (1980); Clinical Chemistry 27:1490-1498 (1981); and Reviews of Infectious Diseases 4:35-68 (1982); and by Kiyotani et al., Hiroshima J. Medical Sciences 33:287-292 (1984); Zbl Bakt Hyg A260-273-285 (1985); Microbiol. Immun. 31:1131-1135 (1987). Despite the availability of these prior NA assays, however, physicians currently still diagnose influenza solely on the basis of symptomology. This is in part due to the fact that these prior assays were complicated and/or required equipment not typically found in a clinical setting. Another shortcoming of these prior assays is that they were unable to discriminate between influenza type. That ability is particularly important to enable physicians to prescribe

the appropriate chemotherapy and/or supportive therapy to combat the infection.

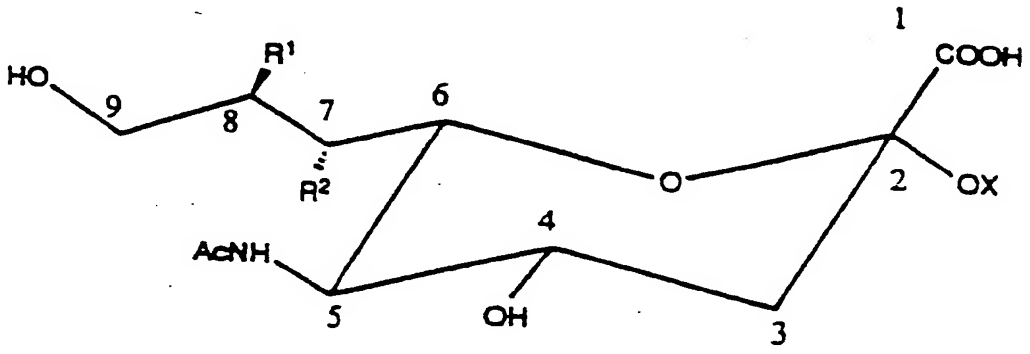
Prior workers have investigated the relationship between the chemical structure of Neu5Ac and its biological function as a substrate for non-influenza NA. Gross, H.J., et al., Biochemistry 27:4279 (1988), examined benzyl-alpha-glycosides of N-acetyl-4-epi-D-neuraminic acid as a substrate for three different bacterial NAs (C. perfringens, A. ureafaciens, and V. cholera) and found significant differences in reactivity. After 22 hrs, the C. perfringens NA cleaved 100% of the substrate while the A. ureafaciens and V. cholera NAs cleaved only 50% and 11% of the substrate, respectively. Kim et al., J. Am. Chem. Soc. 110:6481-6486 described the structural characteristics of substrates accepted by Neu5Ac aldolase, its use in the synthesis of Neu5Ac, and its chemical conversion to the 2-deoxy derivatives, and additionally reported that work was in progress to determine the biological activity of the 2-deoxy derivatives. Brossmer et al., Helv. Chim. Acta 69:2127 (1986); Glycoconjugates 4:145 (1987) reported that the methyl-alpha-glycoside of 4-deoxy Neu5Ac was a good substrate for fowl plague viral Neu5Ac, but not for the three bacterial NAs mentioned above. Additionally, Schauer, R., et al., Eur. J. Biochem. 106:531 (1980), reported that 4-methoxy Neu5Ac was an excellent substrate for fowl plague viral NA but not for V. cholera NA. The 4-methylumbelliferyl derivative of 4-deoxy Neu5Ac is also described in the literature (Helv. Chim. Acta. 69:1927 (1986)). Zbiral et al., Monatsheft fur Chemie 119:127-141 (1988) described the synthesis of 7- and 8-deoxy Neu5Ac. Zbiral et al., Liebigs Ann Chem, 519-526 described the synthesis of the 4-methyl-umbelliferyl-2-α glycosides of 7-epi, 8-epi, 7,8-bis-epi, 8-deoxy, 9-deoxy, and 4,7-dideoxy Neu5Ac and

investigated the behavior of those compounds as inhibitors of the sialidase for V. cholera. Gross, H.J., et al., Eur. J. Biochemistry 106:531 (1987) refers to the 9-azido and 9-fluoro analogs of Neu5Ac and the 7-epi and 7,8-bis-epi analogs of Neu5Ac.

Dislosure of the Invention

One aspect of the invention is a method of detecting human influenza neuraminidase activity in a clinical sample suspected of having such activity comprising:

(a) incubating the sample with a chromogenic modified N-acetylneuraminic acid substrate of the formula:

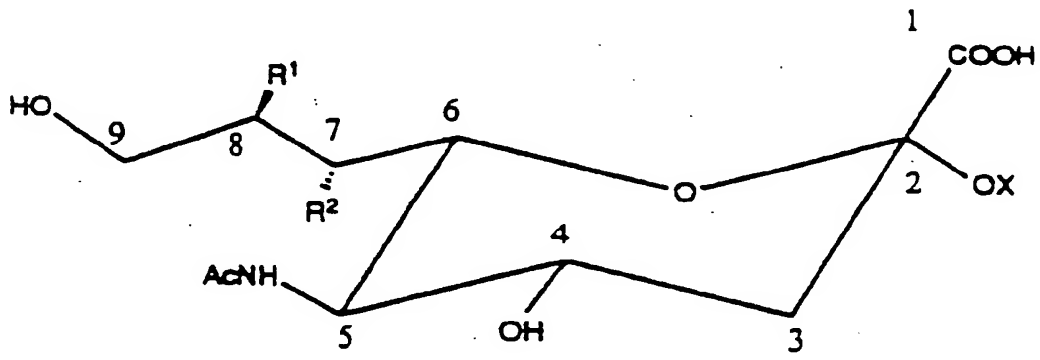


where Ac represents acetyl, R^1 represents hydrogen, fluorine, hydroxy, azido or cyano, R^2 represents hydroxy, hydrogen, fluorine, oxo (=O), or azido with the proviso that one of R^1 and R^2 must be hydroxyl but not both of R^1 and R^2 are hydroxyl, and X represents a chromogenic group that exhibits distinct color when cleaved from the substrate or a salt of said substrate; and

(b) detecting neuraminidase activity by observing whether the sample-substrate mixture exhibits said color after step (a).

Another aspect of the invention is a method of selectively detecting a specific type (e.g., A or B) of human influenza neuraminidase activity in a clinical sample suspected of having human influenza neuraminidase activity from activity exhibited by other types of human influenza neuraminidase comprising:

(a) incubating the sample with a chromogenic modified N-acetylneuraminic acid substrate of the formula:



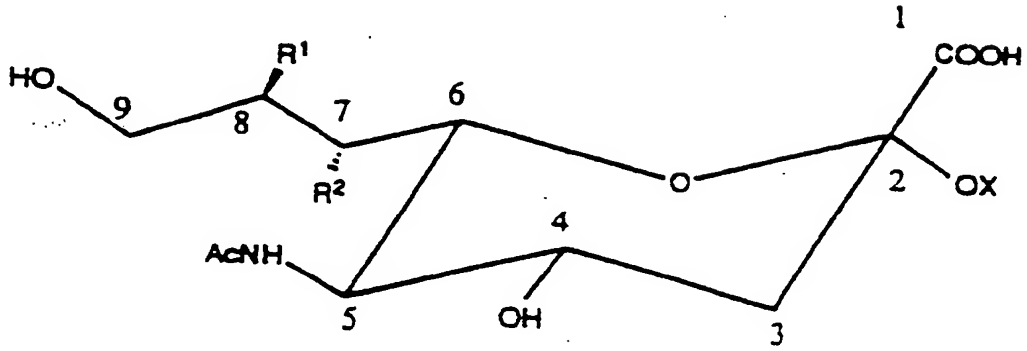
where Ac represents acetyl, R^1 represents hydrogen, fluorine, hydroxy, azido or cyano, R^2 represents hydroxy, hydrogen, fluorine, oxo (=O), or azido with the proviso that one of R^1 and R^2 must be hydroxyl but not both of R^1 and R^2 are hydroxyl, and X represents a chromogenic group that exhibits distinct color when cleaved from the substrate or a salt of said substrate;

(b) observing the color exhibited by the sample-substrate mixture after step (a); and

(c) comparing said color to colors exhibited by activity standards of human influenza neuraminidase of said specific type and other types of human influenza neuraminidase on said substrate.

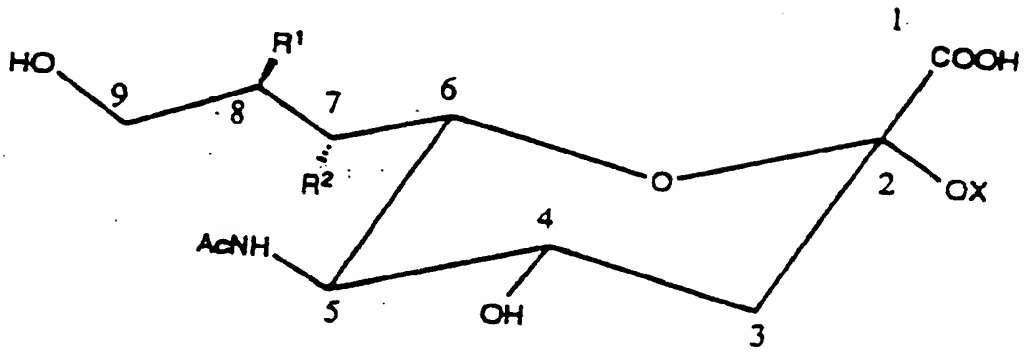
Yet another aspect of the invention is a modified Neu5Ac chromogenic substrate useful for detecting human influenza neuraminidase activity in a

clinical sample suspected of having such activity, said substrate having the formula:



where Ac represents acetyl, R^1 represents fluorine, hydroxy, azido or cyano, R^2 represents hydroxy, hydrogen, fluorine, oxo (=O), or azido with the proviso that one of R^1 and R^2 must be hydroxyl but not both of R^1 and R^2 are hydroxyl, and X is a chromogenic group that exhibits a distinct color when cleaved from the substrate and salts of said substrate.

Still another aspect of the invention is a chromogenic substrate useful for detecting human influenza neuraminidase activity in a clinical sample suspected of having such activity, said substrate having the formula:



where Ac represents acetyl, R^1 represents hydrogen, R2 represents hydroxyl, and X is a chromogenic group selected form the group consisting of 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

Brief Description of the Drawings

In the drawings:

Figure 1 is a schematic diagram depicting the synthesis procedure described in Example 1.

Figure 2 is a schematic diagram depicting the synthesis procedure described in Example 2.

Figure 3 is a schematic diagram depicting the synthesis procedures described in Examples 3 and 5.

Figure 4 is a schematic diagram depicting the synthesis procedure described in Example 4.

Figure 5 is a schematic diagram depicting the synthesis procedure described in Example 6.

Figure 6 is a schematic diagram depicting the synthesis procedure described in Example 7.

Figure 7 is a schematic diagram depicting the synthesis procedure described in Example 8.

Figure 8 is a schematic diagram depicting the synthesis procedure described in Example 9.

Figure 9 is a schematic diagram depicting the synthesis procedure described in Example 10.

Figure 10 is a schematic diagram depicting the synthesis procedure described in Example 11.

Figure 11 is a schematic diagram depicting the synthesis procedure described in Example 12.

Figure 12 is a schematic diagram depicting the synthesis procedure described in Example 13.

Figure 13 is a schematic diagram depicting the synthesis procedure described in Example 14.

Modes for Carrying Out the Invention

The chromogenic modified N-acetylneuraminic acid substrates of the invention and the methods employing them are useful for detecting human influenza neuraminidase activity in clinical samples or specimens and for determining the type of human influenza neuraminidase present in the sample. Accordingly, these substrates and methods are useful for diagnosing influenza infection generally as well as the type of influenza infection present in the human patient from whom the clinical sample was collected. In this regard, the term "influenza" is intended to include influenza types A and B and parainfluenza types 1, 2, and 3. The term "selectively detect" intends the ability to detect NA activity of one type of influenza virus as compared to the activity of other types of influenza virus.

The clinical samples that are tested in the invention will typically be pharyngeal, nasopharyngeal or respiratory secretions collected from patients suffering from influenza as wash, swab, or expectorate specimens. The wash, expectorate, or swab will preferably be combined with an aqueous buffer solution containing a

stabilizer prior to mixing with the substrate. The buffer solution contains a buffer that maintains the pH at about 4 to 7, preferably 5.5 to 6.5, optionally about 0.1% to 10% by weight nonionic detergent, a small amount (1-20 mM) of alkaline earth metal cation (Ca, Mg, preferably Ca), and a sufficient amount of a stabilizer selected from the group consisting of polyhydric sugar alcohols, simple sugars, and disaccharide sugars to enhance the thermal stability of the NA in the sample. The volume of buffer solution combined with the specimen will normally be 0.1 to 2 ml.

The buffer may be organic or inorganic. Examples of suitable buffers are conventional buffers of organic acids and salts thereof such as citrate buffers (e.g. monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), acetate buffers (e.g., acetic acid-sodium acetate mixture), succinate buffers (e.g. succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g. tartaric acid-tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture etc.), fumarate buffers (e.g. fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumaric acid-disodium fumarate mixture), gluconate buffers (e.g. gluconic acid-sodium gluconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium gluconate mixture, etc.) oxalate buffers (e.g. oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g. lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.), acetate buffers (e.g. acetic acid-sodium acetate mixture,

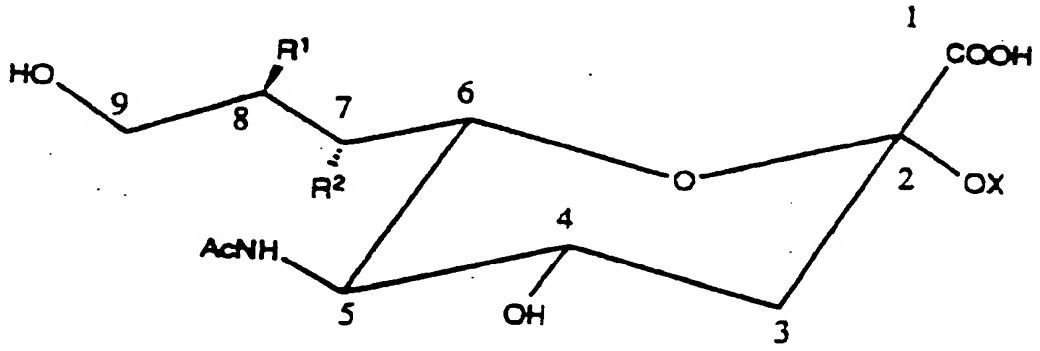
acetic acid-sodium hydroxide mixture, etc.), malate buffers (e.g., D,L-malic acid-disodium malate mixture), phosphate buffers (e.g. monosodium phosphate-disodium phosphate mixture, monosodium phosphate-sodium hydroxide mixture, trisodium phosphate-hydrochloric acid mixture, etc.), 2-(N-morpholino)ethanesulfonc acid, [bis-(2-hydroxyethyl)imino]tris(hydroxymethyl)methane, N-2-acetamidoiminodiacetic acid, 1,3-bis[tris(hydroxymethyl)-methylamino]propane, piperazine-N,N'-bis(2-ethanesulfonic acid), N-2-acetamido-2-aminoethanesulfonic acid, 3-(N-morpholino)-2-hydroxypropanesulfonic acid, N-N-bis-(2-hydroxyethyl)2-aminoethanesulfonic acid, 3-(N-morpholino)propanesulfonic acid, 2-[tris(hydroxymethyl)methylamino]ethanesulfonic acid, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 3-{[tris-(hydroxymethy)methyl]amino}-2-hydroxypropane-sulfonic acid.

Examples of non-ionic detergents useful in the buffer solution are the Pluronics, such as Polysorbate 20 and Polysorbate 80, Triton X-100, NP-40, and alkyl glucosides such as C_8-C_9 alkyl glucoside. The detergent is an optional component and facilitates release of the NA from the viral envelope.

Examples of the stabilizers that are used in the buffer solution are trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol, mannitol, the simple sugars glucose and fructose and the disaccharride sucrose. These polyhydric sugar alcohols, and simple and disaccharride sugars can be used alone or in combination. In order to stabilize the activity of the neuraminidase-containing viruses, the polyhydric sugar alcohols or simple and disaccharride sugars are added to the liquid formulation/excipient system in an amount from 0.2 M to 2.1 M and preferably, 0.6 M to 2.0 M.

Once mixed with the buffer solution, the sample may be stored for prolonged periods, preferably at 2°C to 8°C without significant loss of NA activity.

The substrate that is combined with the buffered, stabilized specimen is a chromogenic Neu5Ac derivative that is modified in the 7- or 8-positions (but not both positions). These substrates may be represented by the following chemical formula:



where R^1, R^2, X and Ac are as defined previously. Preferably X represents 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, nitrophenyl-azophenyl, nitrophenylazoresorcinyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-napthyl or 6-bromo-2-naphthyl. Simple salts of the substrate such as the Na, K, or NH_4^+ salts, may also be used.

As used herein the term "chromogen" is intended to include, without limitation, molecules that exhibit fluorescence. The term "color" is likewise intended to include, without limitation, fluorescence.

Examples of 7- or 8-modified chromogenic Neu5Ac derivatives falling within the above formula are 4-methylumbelliferyl-7-deoxy-N-acetylneuraminic acid-alpha-ketoside, 3-cyanoumbelliferyl-7-deoxy-N-acetylneuraminic acid-alpha-ketoside, 2-nitrophenyl-7-

deoxy-N-acetylneuraminic acid-alphaketoside, 4-nitrophenyl-7-deoxy-N-acetylneuraminic acid-alpha-ketoside, 3-resorufin-7-deoxy-N-acetylneuraminic acid-alpha-ketoside, 5-bromo-4-chloro3-indolyl-7-deoxy-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)-phenyl]-7-deoxy-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)resorcinyl]-7-deoxy-N-acetylneuraminic acid-alpha-ketoside, 3-methoxyphenyl-7-deoxy-N-acetylneuraminic acid-alpha-ketoside, 3-dimethylaminophenyl-7-deoxy-N-acetylneuraminic acid-alpha-ketoside, 4-chloro-1-naphthyl-7-deoxy-N-acetyl-neuraminic acid-alpha-ketoside, 6-bromo-2-naphthyl-7-deoxy-N-acetylneuraminic acid-alpha-ketoside, 4-methylumbelliferyl-7-fluoro-N-acetylneuraminic acid-alpha-ketoside, 3-cyanoumbelliferyl-7-fluoro-N-acetylneuraminic acid-alpha-ketoside, 2-nitrophenyl-7-fluoro-N-acetylneuraminic acid-alpha-ketoside, 4-nitrophenyl-7-fluoro-N-acetylneuraminic acid-alpha-ketoside, 3-resorufin-7-fluoro-N-acetylneuraminic acid-alpha-ketoside, 5-bromo4-chloro-3-indolyl-7-fluoro-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)phenyl]-7-fluoro-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)resorcinyl]-7-fluoro-N-acetylneuraminic acid-alpha-ketoside, 3-methoxyphenyl-7-fluoro-N-acetylneuraminic acid-alpha-ketoside, 3-dimethylaminophenyl-7-fluoro-N-acetyl-neuraminic acid-alpha-ketoside, 4-chloro-1-naphthyl-7-fluoro-N-acetyl-neuraminic acid-alpha-ketoside, 6-bromo-2-naphthyl-7-fluoro-N-acetylneuraminic acid-alpha-ketoside, 4-methylumbelliferyl-7-azido-N-acetylneuraminic acid-alpha-ketoside, 3-cyanoumbelliferyl-7-azido-N-acetylneuraminic acid-alpha-ketoside, 2-nitrophenyl-7-azido-N-acetylneuraminic acid-alpha-ketoside, 4-nitrophenyl-7-azido-N-acetylneuraminic acid-alpha-ketoside, 3-resorufin-7-azido-N-acetylneuraminic acid-alpha-ketoside, 5-bromo4-chloro-

3-indolyl-7-azido-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)phenyl]-7-azido-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)resorcinyl]-7-azido-N-acetylneuraminic acid-alpha-ketoside, 3-methoxyphenyl-7-azido-N-acetylneuraminic acid-alpha-ketoside, 3-dimethylaminophenyl-7-azido-N-acetylneuraminic acid-alpha-ketoside, 4-chloro-1-naphthyl-7-azido-N-acetylneuraminic acid-alpha-ketoside, 6-bromo-2-naphthyl-7-azido-N-acetylneuraminic acid-alpha-ketoside, 4-methylumbelliferyl-7-keto-N-acetylneuraminic acid-alpha-ketoside, 3-cyanoumbelliferyl-7-keto-N-acetylneuraminic acid-alpha-ketoside, 2-nitrophenyl-7-keto-N-acetylneuraminic acid-alpha-ketoside, 4-nitrophenyl-7-keto-N-acetylneuraminic acid-alpha-ketoside, 3-resorufin-7-keto-N-acetylneuraminic acid-alphaketoside, 5-bromo-4-chloro-3-indolyl-7-keto-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)phenyl]-7-keto-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)resorcinyl]-7-keto-N-acetylneuraminic acid-alpha-ketoside, 3-methoxyphenyl-7-keto-N-acetylneuraminic acid-alpha-ketoside, 3-dimethylaminophenyl-7-keto-N-acetylneuraminic acid-alpha-ketoside, 4-chloro-1-naphthyl-7-azido-N-acetylneuraminic acid-alpha-ketoside, 6-bromo-2-naphthyl-7-keto-N-acetylneuraminic acid-alpha-ketoside, 4-methylumbelliferyl-8-deoxy-N-acetylneuraminic acid-alpha-ketoside, 3-cyanoumbelliferyl-8-deoxy-Nacetylneuraminic acid-alpha-ketoside, 2-nitrophenyl-8-deoxy-N-acetylneuraminic acid-alpha-ketoside, 4-nitrophenyl-8-deoxy-N-acetylneuraminic acid-alphaketoside, 3-resorufin-8-deoxy-N-acetylneuraminic acid-alpha-ketoside, 5-bromo-4-chloro-3-indolyl8-deoxy-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)phenyl]-8-deoxy-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)resorcinyl]-8-deoxy-N-acetylneuraminic acid-

alphaketoside, 3-methoxyphenyl-8-deoxy-N-acetylneuraminic acid-alpha-ketoside, 3-dimethylaminophenyl-8-deoxy-N-acetylneuraminic acid-alpha-ketoside, 4-chloro-1-naphthyl-7-azido-N-acetylneuraminic acid-alpha-ketoside, 6-bromo-2-naphthyl-8-deoxy-N-acetylneuraminic acid-alpha-ketoside, 4-methylumbelliferyl-8-fluoro-N-acetylneuraminic acid-alpha-ketoside, 3-cyanoumbelliferyl-8-fluoro-N-acetylneuraminic acid-alpha-ketoside, 2-nitrophenyl-8-fluoro-N-acetylneuraminic acid-alpha-ketoside, 4-nitrophenyl-8-fluoro-N-acetylneuraminic acid-alphaketoside, 3-resorufin-8-fluoro-N-acetylneuraminic acid-alpha-ketoside, 5-bromo-4-chloro-3-indolyl-8-fluoro-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)phenyl]-8-fluoro-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)resorcinyl]-8-fluoro-N-acetylneuraminic acid-alpha-ketoside, 3-methoxyphenyl-8-fluoro-N-acetylneuraminic acid-alpha-ketoside, 3-dimethylaminophenyl-8-fluoro-N-acetylneuraminic acid-alpha-ketoside, 4-chloro-1-naphthyl-7-azido-N-acetylneuraminic acid-alpha-ketoside, 6-bromo-2-naphthyl-8-fluoro-N-acetylneuraminic acid-alpha-ketoside, 4-methylumbelliferyl-8-azido-N-acetylneuraminic acid-alpha-ketoside, 3-cyanoumbelliferyl8-azido-N-acetylneuraminic acid-alpha-ketoside, 2-nitrophenyl-8-azido-N-acetylneuraminic acid-alpha-ketoside, 4-nitrophenyl-8-azido-N-acetylneuraminic acid-alphaketoside, 3-resorufin-8-azido-N-acetylneuraminic acid-alpha-ketoside, 5-bromo-4-chloro-3-indolyl-8-azido-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)phenyl]-8-azido-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)resorcinyl]-8-azido-N-acetylneuraminic acid-alphaketoside, 3-methoxyphenyl-8-azido-N-acetylneuraminic acid-alpha-ketoside, 3-dimethylaminophenyl-8-azido-N-acetylneuraminic acid-alpha-ketoside, 4-chloro-1-naphthyl-7-azido-N-acetyl-

neuraminic acid-alpha-ketoside, 6-bromo-2-naphthyl-8-azido-N-acetylneuraminic acid-alpha-ketoside, 4-methylumbelliferyl-8-cyano-N-acetylneuraminic acid-alpha-ketoside, 3-cyanoumbelliferyl-8-cyanoN-acetylneuraminic acid-alpha-ketoside, 2-nitrophenyl-8-cyano-N-acetylneuraminic acid-alpha-ketoside, 4-nitrophenyl-8-cyano-N-acetylneuraminic acid-alphaketoside, 3-resorufin-8-cyano-N-acetylneuraminic acid-alpha-ketoside, 5-bromo-4-chloro3-indolyl-8-cyano-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)phenyl]-8-cyano-N-acetylneuraminic acid-alpha-ketoside, 2-[4-(4-nitrophenylazo)resorcinyl]-8-cyano-N-acetylneuraminic acid-alphaketoside, 3-methoxyphenyl-8-cyano-N-acetylneuraminic acid-alpha-ketoside, 3-dimethylaminophenyl-8-cyano-N-acetylneuraminic acid-alpha-ketoside, 4-chloro-1-naphthyl-7-azido-N-acetylneuraminic acid-alpha-ketoside and 6-bromo-2-naphthyl-8-cyano-N-acetylneuraminic acid-alpha-ketoside.

The above-described Neu5Ac derivatives are generally made by protecting the functional groups of Neu5Ac at the 1, 2, 4, 7 or 8 (as the case may be), and 9 positions, modifying the 7 or 8 position as indicated, deprotecting the other positions, and coupling the 7- or 8-modified Neu5Ac with the chromogen. Details of these reactions are provided in the Examples, infra. The Neu5Ac derivatives modified in the 7- or 8- positions with azido or cyano groups may be produced as epimeric mixtures due to the mechanism of the reactor involved coupled with the configuration of neighboring group(s). The epimeric mixture may be used or the epimers may be separated and used separately.

The substrate will normally be added to the buffered, stabilized sample in amounts ranging between 0.05 mM and 0.5 mM. The mixture is incubated at ambient temperature to physiological temperature (i.e., about

22°C to 37°C) for a time sufficient to permit any NA in the sample to react with the substrate. That time will normally be in the range of 20 to 120 minutes, more usually 30 to 60 minutes. If there is NA activity in the sample, the chromogenic group will be cleaved from the substrate and the liberated chromogen will impart a characteristic color to the mixture. Since the substrates of the invention may exhibit different reactivity to the different human influenza NAs, the specific type of influenza infection may be determined by comparing the color of the sample mixture with the color of standard reaction mixtures for each influenza NA type. For instance, influenza A may be distinguished from influenza B on the basis of substrate reactivity with the NAs of these influenza viruses. The following table indicates the color generated when NA reacts with a modified Neu5Ac and releases the chromogen.

Released Chromogen	Type of Detection	Color
5-bromo-4-chloro-3-indolol	colorimetric/ visual	blue/purple in the presence of nitroblue tetrazolium
4-methyl-umbelliferone	fluorometric	fluorescent emission at 450 nm after excitation at 360 nm
3-cyanoumbelliferone	fluorometric	fluorescent emission at 454 nm after excitation at 415 nm

resorufin	colorimetric/ visual	pink/red
2-nitrophenol	colorimetric/ visual	yellow
4-nitrophenol	colorimetric/ visual	yellow
nitrophenylazo-phenol	colorimetric/ visual	orange
nitrophenylazo-resorcinol	colorimetric/ visual	green blue (presence of Mg^{++})
3-methoxyphenol	colorimetric/ visual	red to blue after reaction with diazonium salt
3-dimethyl-aminophenol	colorimetric/ visual	red to blue after reaction with diazonium salt
6-bromo-2-naphthol	colorimetric/ visual	red to blue after reaction with diazonium salt

4-chloro-1-naphthol	colorimetric/ visual	red to blue after reaction with diazonium salt

Accordingly, the present invention provides a simple and rapid technique for selectively diagnosing influenza that may be carried out in the clinic or physician's office and enable the physician to prescribe the appropriate therapy to treat the infection and/or the appropriate prophylactic treatment to persons in close contact with the infected patient.

The invention is further illustrated by the following examples. These examples are not intended to limit the invention in any manner.

Examples

1. Synthesis of 7-Keto Neu5Ac

The synthesis scheme for this compound is shown in Figure 1.

N-acetylneuraminic acid is protected as the methyl ester methyl ketoside (Neu5Ac-MEMK) by treatment with methanol under Dowex 50W ion-exchange resin acid catalysis. Subsequent reaction with acetone and catalytic p-toluenesulfonic acid for 4 hr affords the 8,9-isopropylidene adduct. This intermediate is treated with one equivalent of t-butyldimethylsilyl (TBDMS) chloride, imidazole and a catalytic amount of dimethylaminopyridine to selectively yield the 4-silylated derivative. Oxidation of the lone 7-alcoholic group with pyridinium-dichromate gives the 7-keto adduct. Saponification of the ester and deprotection of the TBDMS group and acid-labile ketal and

ketoside groups is then accomplished with hydroxide solution, followed by tetrabutylammonium fluoride and dilute acid to afford 7-keto-N-acetylneuraminic acid.

2. Synthesis of Chromogenic 7-Keto Neu5Ac

The synthesis scheme for this compound is shown in Figure 2.

The 8,9-isopropylidene-4-TBDMS-7-keto Neu5Ac-MEMK intermediate from the previous synthesis is treated with dilute hydrochloric acid solution to deprotect the 4,8 and 9 positions and the resulting free acid esterified with methanol/trifluoroacetic acid. Formation of the glycosyl chloride with concomitant acetylation of all free OH groups is accomplished by treatment in excess acetyl chloride overnight. Coupling of this intermediate with the sodium salt of nitrophenylazoresorcinol (NAR) is done in dimethylformamide (DMF) solution (2 hr). The final product, 2-[4-(4-nitrophenylazo)resorcinyl]-7-keto-N-acetylneuraminic acid-alpha-ketoside (sodium salt), is obtained by deprotecting the alcohol groups with methoxide ion and saponification of the methyl ester under base catalysis.

3. Synthesis of 7-Azido Neu5Ac

The synthesis scheme for this compound is shown in Figure 3.

8,9-isopropylidene protected Neu5Ac-MEMK is formed according to the procedure detailed in Example 1. Treatment with 1 eq. TBDMS-Cl, imidazole and 4-dimethylaminopyridine in DMF at 65-70°C affords 8,9-isopropylidene-4-O-TBDMS Neu5Ac methyl ester methyl ketoside. Treatment of this compound with triethylamine and methanesulfonyl (Ms) chloride in methylene chloride forms the 8,9-isopropylidene-4-O-TBDMS-7-Ms Neu5Ac methyl ester methyl ketoside. Reaction of this compound with

sodium azide in methyl ethyl ketone at 100°C will form the corresponding 7-azido-8,9-isopropylidene-4-O-TBDMS Neu5Ac. Deprotection of this compound will consist of treatment with sodium hydroxide followed by Dowex-50W (H^+) then treatment with tetrabutylammonium fluoride in THF to remove the silyl group, and finally treatment with dilute HCl/Dowex-50W (H^+) to afford 7-azido-N-acetylneuraminic acid.

4. Synthesis of Chromogenic 7-Azido Neu5Ac

The synthesis scheme for this compound is depicted in Figure 4.

Neu5Ac methyl ester is formed through the usual route, as is the glycosyl chloride which is coupled to the sodium salt of resorufin in DMF (2 hr). Deprotection (deacetylation) is accomplished by treatment with sodium ethoxide in methanol. The 7-azido group is then introduced. First the 8 and 9-hydroxy groups will be protected as the isopropylidene by treatment with excess acetone and a catalytic amount of p-toluenesulfonic acid at room temperature. The 4-hydroxy group will then be protected as the O-TBDMS by treatment with 5 equivalents of imidazole, 1 equivalent of t-buyldimethylsilyl chloride, and a catalytic amount of dimethylaminopyridine in DMF, at 65°C. The 7-hydroxy will then be mesylated by treating the compound with methanesulfonyl chloride and triethylamine in methylene chloride at 0°C. The azido group will be substituted on the 7-position by treating the mesylate with sodium azide at 100°C. The molecule will then be fully deprotected by treating with p-toluenesulfonic acid, tetrabutylammonium fluoride, and finally sodium hydroxide to give 2-(3-resorufin)-7-azido-N-acetylneuraminic acid.

5. Synthesis of 7-Deoxy Neu5Ac

The synthesis scheme for this compound is also shown in Figure 3.

4-O-TBDMS-7-mesyl-8,9-isopropylidene Neu5Ac methyl ester methyl ketoside is prepared according to the procedure given above. Treatment of this compound in sodium borohydride in DMF gives the reduction product. Full deprotection by treatment with sodium hydroxide followed by Dowex-50W (H^+), then treatment with tetrabutylammonium fluoride in THF, and finally treatment with dilute HCl/Dowex-50W (H^+) affords 7-deoxy-N-acetylneuraminic acid.

6. Synthesis of Chromogenic 7-Deoxy Neu5Ac

The synthesis scheme for this compound is shown in Figure 5.

7-deoxy Neu5Ac is converted to its methyl ester and then to the peracetylated glycosyl chloride using excess acetyl chloride overnight. Coupling for 2 hr with the sodium salt of resorufin takes place in DMF. The coupled product will then be deprotected by treatment with sodium methoxide in methanol followed by sodium hydroxide to form the sodium salt of 2-(3-resorufin)-7-deoxy-N-acetylneuraminic acid.

7. Synthesis of 8-Azido Neu5Ac

The synthesis scheme for this compound is shown in Figure 6.

N-acetylneuraminic acid is protected as Neu5Ac-MEMK, after which the 4,9-disilylated intermediate is obtained by treatment with 2 equivalents of TBDMS-Cl with imidazole/dimethylaminopyridine in DMF. The more reactive 8-alcohol group is tosylated with tosyl chloride/pyridine (5°C for 7 hr) and subsequently displaced by sodium azide in acetone at 100°C. The

desired compound, 8-azido-N-acetylneuraminic acid, is obtained after deprotection with base, fluoride ion and dilute acid.

8. Synthesis of Chromogenic 8-Azido Neu5Ac

The synthesis scheme for this compound is shown in Figure 7.

Neu5Ac methyl ester is prepared by treating with methanol under trifluroacetic acid catalysis and converted to the glycosyl chloride in excess acetyl chloride. Coupling with 5-bromo-4-chloro-3-indolol is done in DMF with 1 equivalent of sodium hydroxide. Protection of the 4 and 9-alcohol groups is done with 2.5 eq. of TBDMS-Cl, imidazole and catalytic dimethylaminopyridine. Tosylation of the 8-alcohol group and displacement with azide ion is performed as described previously. The desired product, 2-[3-(4-chloro-5-bromo)-indolyl]-8-azido-N-acetylneuraminic acid-alpha-ketoside (sodium salt), is obtained after deprotection with base, fluoride ion and dilute acid.

9. Synthesis of 8-Cyano Neu5Ac

The synthesis scheme for this compound is shown in Figure 8.

The 8-tosylated 4,9-di-O-TBDMS Neu5Ac-MEMK is prepared as in the synthesis of 8-azido Neu5Ac. The tosyl group is displaced with sodium cyanide in acetone (100°C) to give the 8-cyano derivative. The final desired product, 8-cyano-N-acetylneuraminic acid, is obtained after deprotection with base, fluoride ion and dilute acid.

10. Synthesis of Chromogenic 8-Cyano Neu5Ac

The synthesis scheme for this compound is shown in Figure 9.

The synthesis of 5-bromo-4-chloro-3-indolyl-8-cyano Neu5Ac is done in the same manner as the corresponding 5-bromo-4-chloro-3-indolyl-8-azido Neu5Ac (see Example 8), only the displacement reaction is done with sodium cyanide rather than sodium azide.

11. Synthesis of 8-Deoxy Neu5Ac

The synthesis scheme for this compound is shown in Figure 10.

This compound is prepared in an identical manner to that of 8-azido Neu5Ac or 8-cyano Neu5Ac, only the 8-tosyl intermediate is reduced with sodium borohydride (4 hr) in dimethylsulfoxide to give the corresponding deoxy derivative. The final product, 8-deoxy-N-acetylneuraminic acid, is obtained after deprotection with base, fluoride ion and dilute acid.

12. Synthesis of Chromogenic 8-Deoxy Neu5Ac

The synthesis scheme for this compound is shown in Figure 11.

The 4,9-di-O-TBDMS-8-deoxy Neu5Ac-MEMK intermediate is deprotected with dilute hydrochloric acid and the free acid reesterified with methanol/trifluoroacetic acid. As before, the corresponding glycosyl chloride peracetate is formed by treatment in excess acetyl chloride and coupled with the sodium salt of nitrophenylazophenol in DMF. Standard base and acid deprotection affords the adduct 2-[4-(4-nitrophenylazo)-phenyl]-8-deoxy-N-acetylneuraminic acid-alpha-ketoside (sodium salt).

13. Synthesis of 7-Fluoro Neu5Ac

The synthesis scheme for this compound is depicted in Figure 12.

Neu5Ac-MEMK 8,9-isopropylidene is treated with one equivalent of tert-butyldimethylsilyl chloride to obtain the corresponding 4-TBDMS derivative. Oxidation with PDC affords the 7-keto compound. Reduction with borane-ammonia gives primarily the 7-epimeric alcohol which may then be converted to the corresponding 7-fluoride with DAST (original stereochemistry). Deprotection with dilute base and acid will afford 7-fluoro Neu5Ac after chromotography on Dowex 1 (formate form) or cellulose.

14. Synthesis of Chromogenic 7-Fluoro Neu5Ac

The synthesis scheme for this compound is depicted in Figure 13.

The synthesis of 4-chloro-1-naphthyl-7-flouro Neu5Ac is done in the same manner as in Example 12 except that the sodium salt of 4-chloro-1-naphthol is used.

15. Enzymatic testing of 7-epi-Neu5Ac

50 µl of an influenza virus was mixed with a reaction mixture containing 50 µl of the substrate 4-methylumbelliferyl Neu5Ac at various concentrations in the submillimolar to millimolar range, 150 µl of the inhibitor 7-epi-Neu5Ac at various concentrations in the submillimolar to millimolar range, and 50 µl of 100 mM $CaCl_2$. All solutions were made up in a 50 mM sodium acetate buffer, pH 5.9. After incubation at 37°C for 15 to 30 minutes (depending on virus strain), the reaction was terminated by adding 500 µl of 1 M Tris, pH 9.0, with 1.33% ethanol. The fluorescence intensity was measured at an excitation wavelength of 360 nm and an emission wavelength of 450 nm with a fluorescence spectro-photometer (Hitachi Model 3010). 4-methylumbelliferone

in 1 M Tris, pH 9.0, with 1.33% ethanol served as a standard. Enzyme activity was expressed as mM of Neu5Ac liberated per minute per 50 µl of virus. A plot of 1/v vs. 1/[S] for varying concentrations of substrate and inhibitor showed typical competitive inhibition. Plotting the slopes of the 1/v vs. 1/[S] plot versus the inhibitor concentration allowed for the calculation of K_i for 7-epi-Neu5Ac as follows:

Virus Subtype	K_i (mM)
Influenza A (H1N1)	3.408
Influenza A (H3N2)	24.83
Influenza B	1.346

(The native substrate, Neu5Ac, had a K_i=0.626 mM when the Influenza A (H1N1) virus was used.)

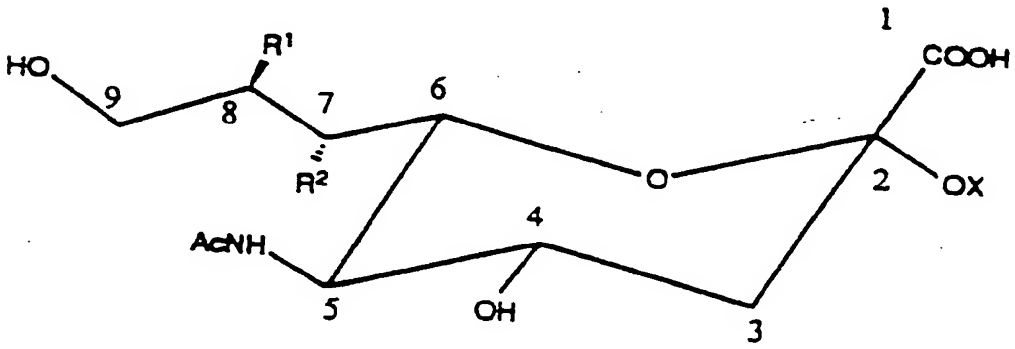
The K_i for 7-epi-Neu5Ac indicates how the compound interacts with the enzyme as well as the rate at which it interacts. In general, the lower the K_i, the greater the degree of inhibition at any given substrate and inhibitor concentration. It is also desirable to have a modified compound which can interact with an enzyme in a similar manner as the native compound without compromising its ability as a substrate. The K_i gives a first indication of the compound's interaction with the enzyme.

Modifications of the above-described modes for carrying out the invention that are obvious to those of skill in the fields of organic chemistry, virology, biochemistry, medical diagnostics, and related fields are intended to be within the scope of the following claims.

Claims

1. A method of detecting human influenza neuraminidase activity in a clinical sample suspected of having such activity comprising:

(a) incubating the sample with a chromogenic modified N-acetylneuraminic acid substrate of the formula:



where Ac represents acetyl, R^1 represents hydrogen, fluorine, hydroxy, azido or cyano, R^2 represents hydroxy, hydrogen, fluorine, oxo, or azido with the proviso that one of R^1 and R^2 must be hydroxyl but not both of R^1 and R^2 are hydroxyl, and X represents a chromogenic group that exhibits distinct color when cleaved from the substrate or a salt of said substrate; and

(b) detecting neuraminidase activity by observing whether the sample-substrate mixture exhibits said color after step (a).

2. The method of claim 1 wherein the clinical sample is a pharyngeal, nasopharyngeal or respiratory secretion.

3. The method of claim 1 or 2 wherein R^1 represents hydrogen, R^2 represents hydroxy, and X is

selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

4. The method of claim 1 or 2 wherein R^1 represents fluorine, R^2 represents hydroxy, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

5. The method of claim 1 or 2 wherein R^1 represents azido, R^2 represents hydroxy, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

6. The method of claim 1 or 2 wherein R^1 represents cyano, R^2 represents hydroxy, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

7. The method of claim 1 or 2 wherein R^1 represents hydroxy, R^2 represents hydrogen, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

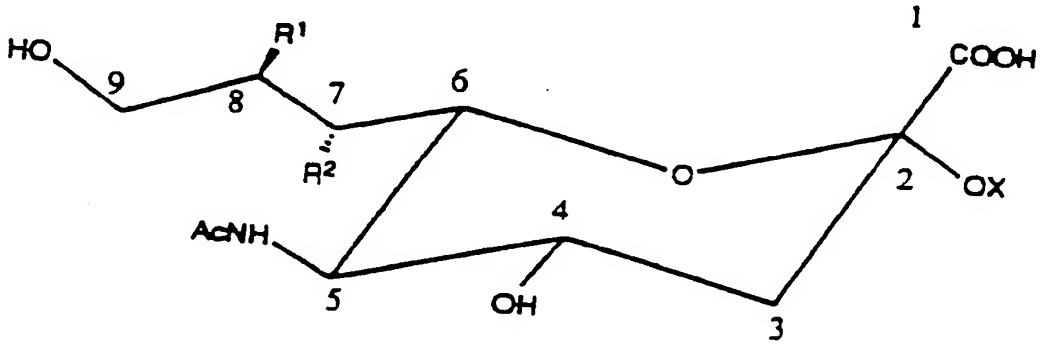
8. The method of claim 1 or 2wherein R^1 represents hydroxy, R^2 represents fluorine, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

9. The method of claim 1 or 2 wherein R^1 represents hydroxy, R^2 represents oxo, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

10. The method of claim 1 or 2 wherein R^1 represents hydroxy, R^2 represents azido, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

11. A method of selectively detecting a specific type of human influenza neuraminidase activity in a clinical sample suspected of having human influenza neuraminidase activity comprising:

(a) incubating the sample with a chromogenic modified N-acetylneuraminic acid substrate of the formula:



where Ac represents acetyl, R^1 represents hydrogen, fluorine, hydroxy, azido or cyano, R^2 represents hydroxy, hydrogen, fluorine, oxo, or azido with the proviso that one of R^1 and R^2 must be hydroxyl but not both of R^1 and R^2 are hydroxyl, and X represents a chromogenic group that exhibits distinct color when cleaved from the substrate or a salt of said substrate;

(b) observing the color exhibited by the sample-substrate mixture after step (a); and

(c) comparing said color to colors exhibited by activity standards of human influenza neuraminidase of said specific type and other types of human influenza neuraminidase on said substrate.

12. The method of claim 11 wherein the specific type of human influenza neuraminidase activity is human influenza A neuraminidase activity or influenza B

neuraminidase activity, or parainfluenza neuraminidase activity.

13. The method of claim 11 or 12 wherein the clinical sample is a pharyngeal, nasopharyngeal, or respiratory secretion.

14. The method of claim 11, 12 or 13 wherein R^1 represents hydrogen, R^2 represents hydroxy, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

15. The method of claim 11, 12 or 13 wherein R^1 represents fluorine, R^2 represents hydroxy, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

16. The method of claim 11, 12 or 13 wherein R^1 represents azido, R^2 represents hydroxy, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

17. The method of claim 11, 12 or 13 wherein R^1 represents cyano, R^2 represents hydroxy, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

18. The method of claim 11, 12 or 13 wherein R^1 represents hydroxy, R^2 represents hydrogen, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

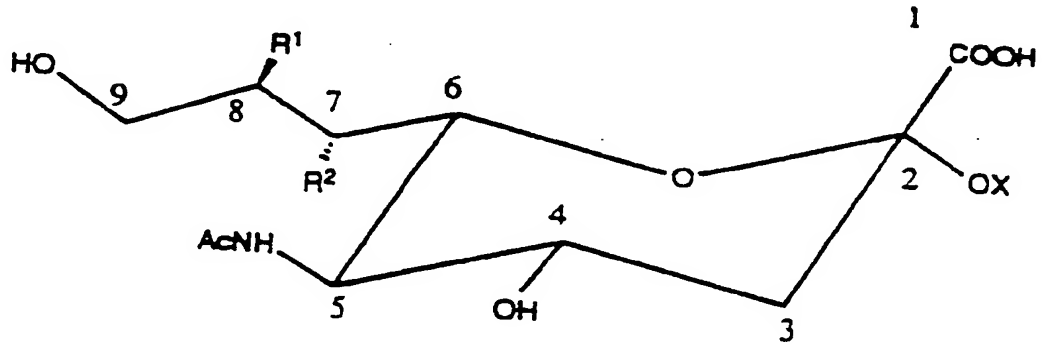
19. The method of claim 11, 12 or 13 wherein R^1 represents hydroxy, R^2 represents fluorine, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

20. The method of claim 11, 12 or 13 wherein R^1 represents hydroxy, R^2 represents oxo, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl,

3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

21. The method of claim 11, 12 or 13 wherein R^1 represents hydroxy, R^2 represents azido, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

22. A chromogenic substrate useful for detecting human influenza neuraminidase activity in a clinical sample suspected of having such activity, said substrate having the formula:



where Ac represents acetyl, R^1 represents hydrogen, fluorine, hydroxy, azido or cyano, R^2 represents hydroxy, hydrogen, fluorine, oxo, or azido with the proviso that one of R^1 and R^2 must be hydroxyl but not both of R^1 and R^2 are hydroxyl, and X is a chromogenic group that exhibits a distinct color when cleaved from the substrate and salts of said substrate.

23. The chromogenic substrate of claim 22 wherein R^1 represents fluorine, R^2 represents hydroxy, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

24. The chromogenic substrate of claim 22 wherein R^1 represents azido, R^2 represents hydroxy, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

25. The chromogenic substrate of claim 22 wherein R^1 represents cyano, R^2 represents hydroxy, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

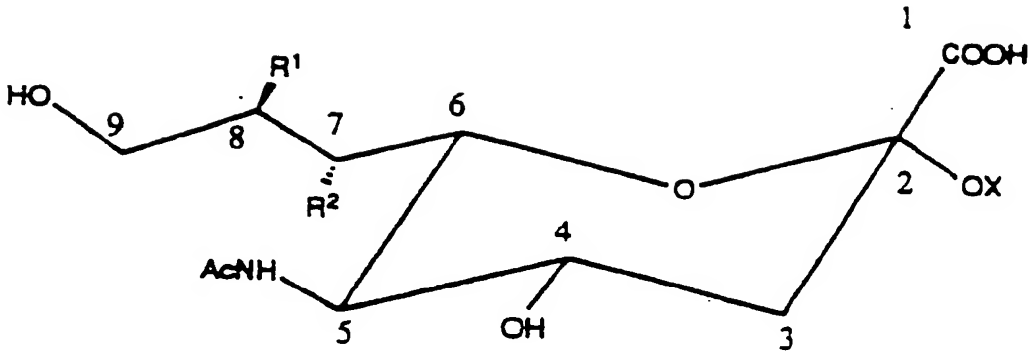
26. The chromogenic substrate of claim 22 wherein R^1 represents hydroxy, R^2 represents hydrogen, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

27. The chromogenic substrate of claim 22 wherein R^1 represents hydroxy, R^2 represents fluorine, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

28. The chromogenic substrate of claim 22 wherein R^1 represents hydroxy, R^2 represents oxo, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

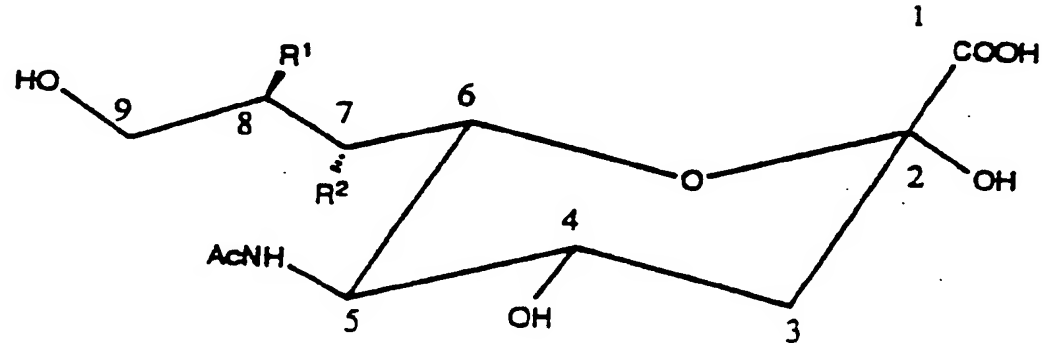
29. The chromogenic substrate of claim 22 wherein R^1 represents hydroxy, R^2 represents azido, and X is selected from the group consisting of 4-methylumbelliferyl, 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl.

30. A chromogenic substrate useful for detecting human influenza neuraminidase activity in a clinical sample suspected of having such activity, said substrate having the formula:



where Ac represents acetyl, R^1 represents hydrogen, R^2 represents hydroxy, and X is selected from the group consisting of 3-cyanoumbelliferyl, 2-nitrophenyl, 4-nitrophenyl, 3-resorufin, 5-bromo-4-chloro-3-indolyl, 4-nitrophenylazoresorcinyl, 4-nitrophenylazophenyl, 3-methoxyphenyl, 3-dimethylaminophenyl, 4-chloro-1-naphthyl and 6-bromo-2-naphthyl, and salts of said substrate.

31. A modified N-acetylneuraminic acid having the formula:



where Ac represents acetyl, R^1 represents fluorine, hydroxy, azido or cyano, R^2 represents hydroxy, fluorine, oxo or azido with the proviso that one of R^1 and R^2 must be hydroxyl but not both R^1 and R^2 are hydroxyl.

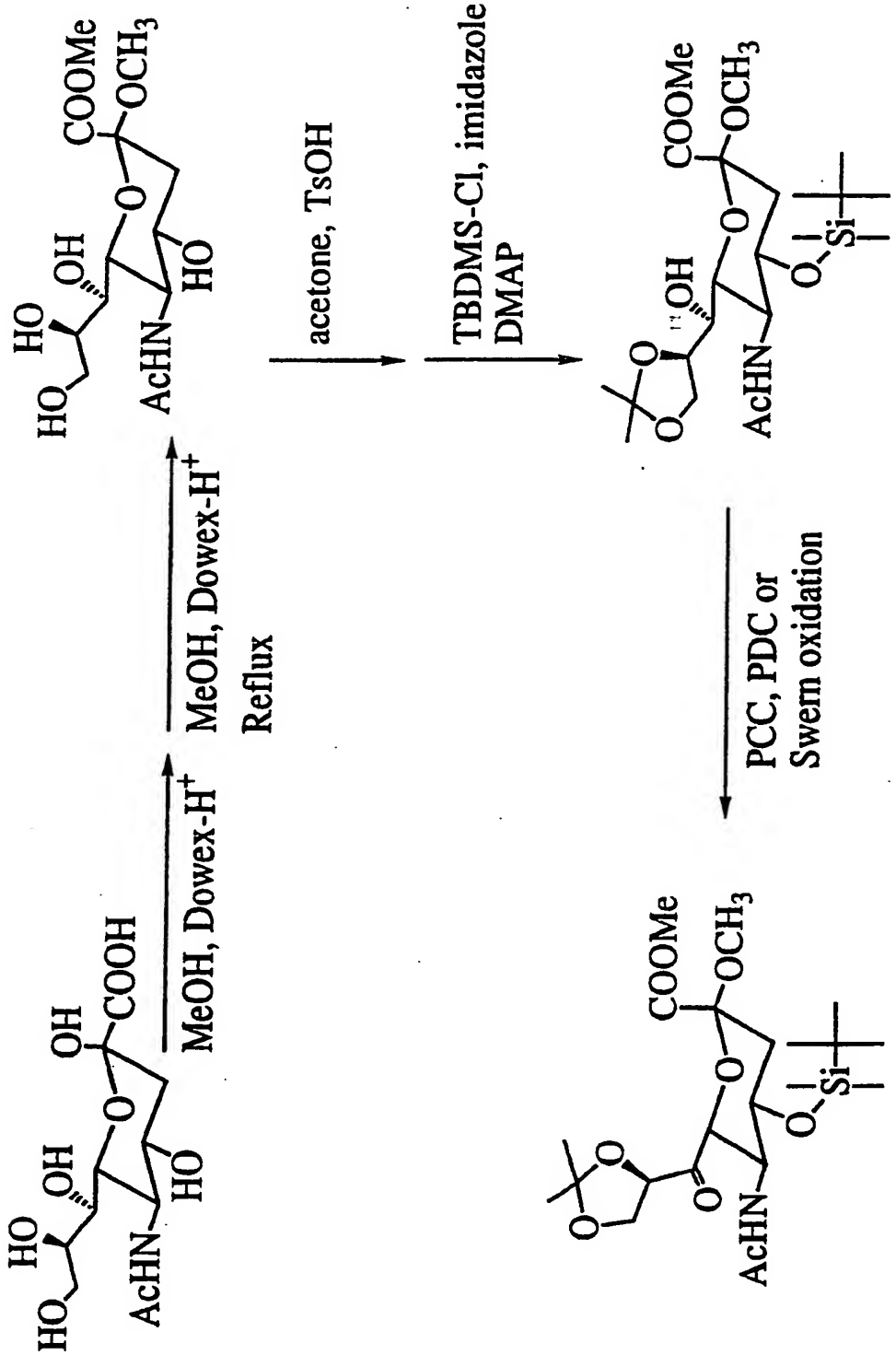
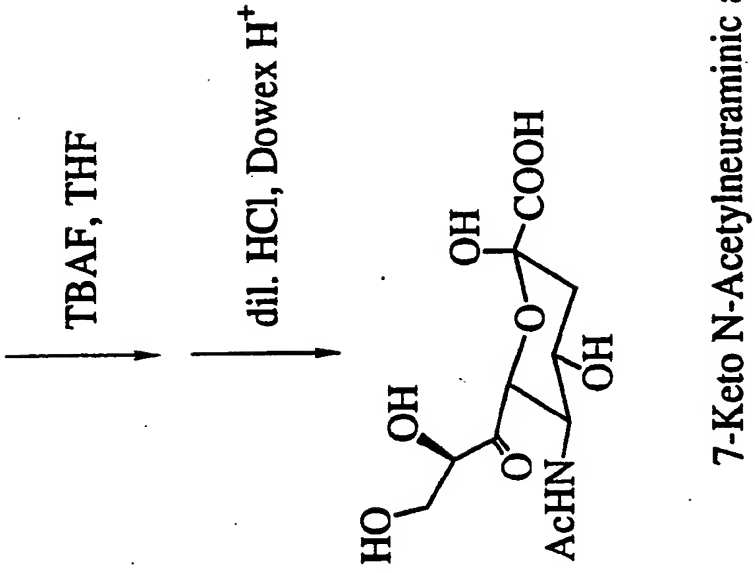


Figure 1A



7-Keto N-Acetylneuraminic acid

Figure 1B

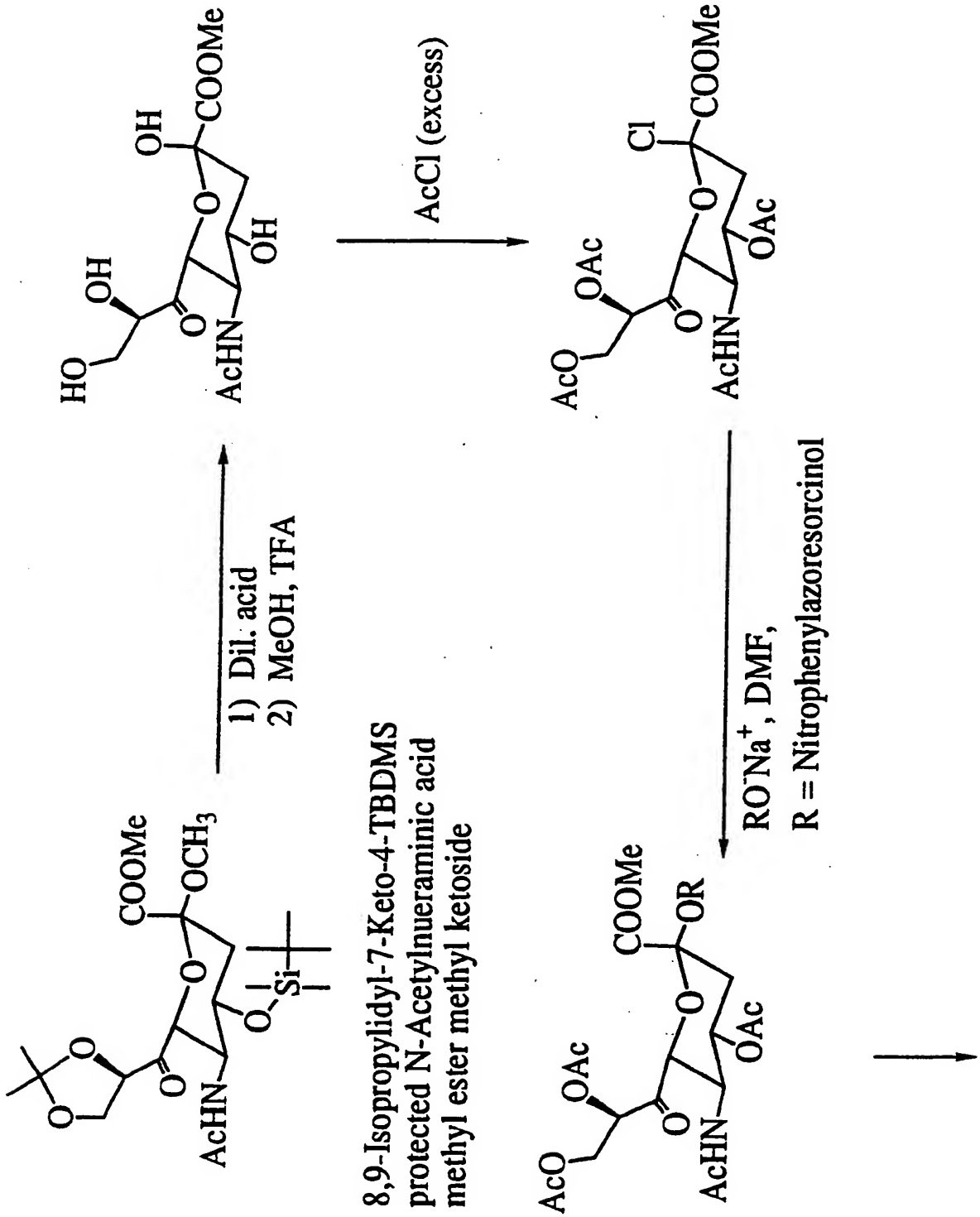
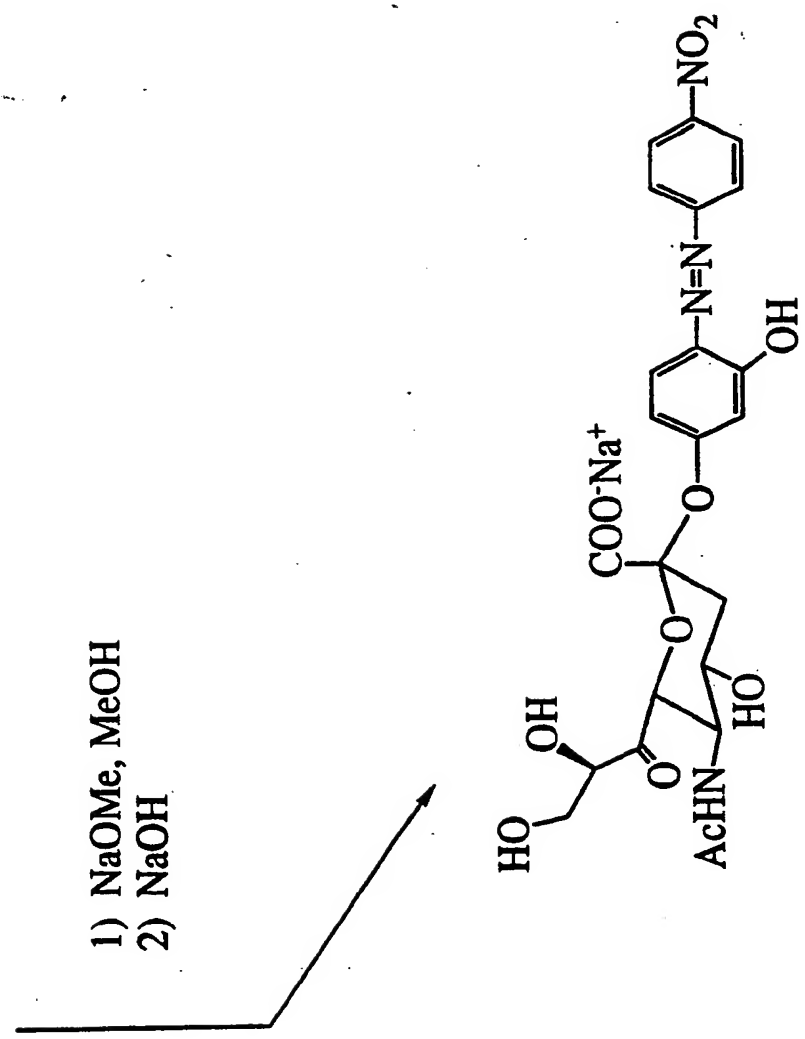


Figure 2A



2-{4-(4-Nitrophenylazo)resorcinol}-7-Keto-N-Acetylneuraminic acid
-α-ketoside (sodium salt)

Figure 2B

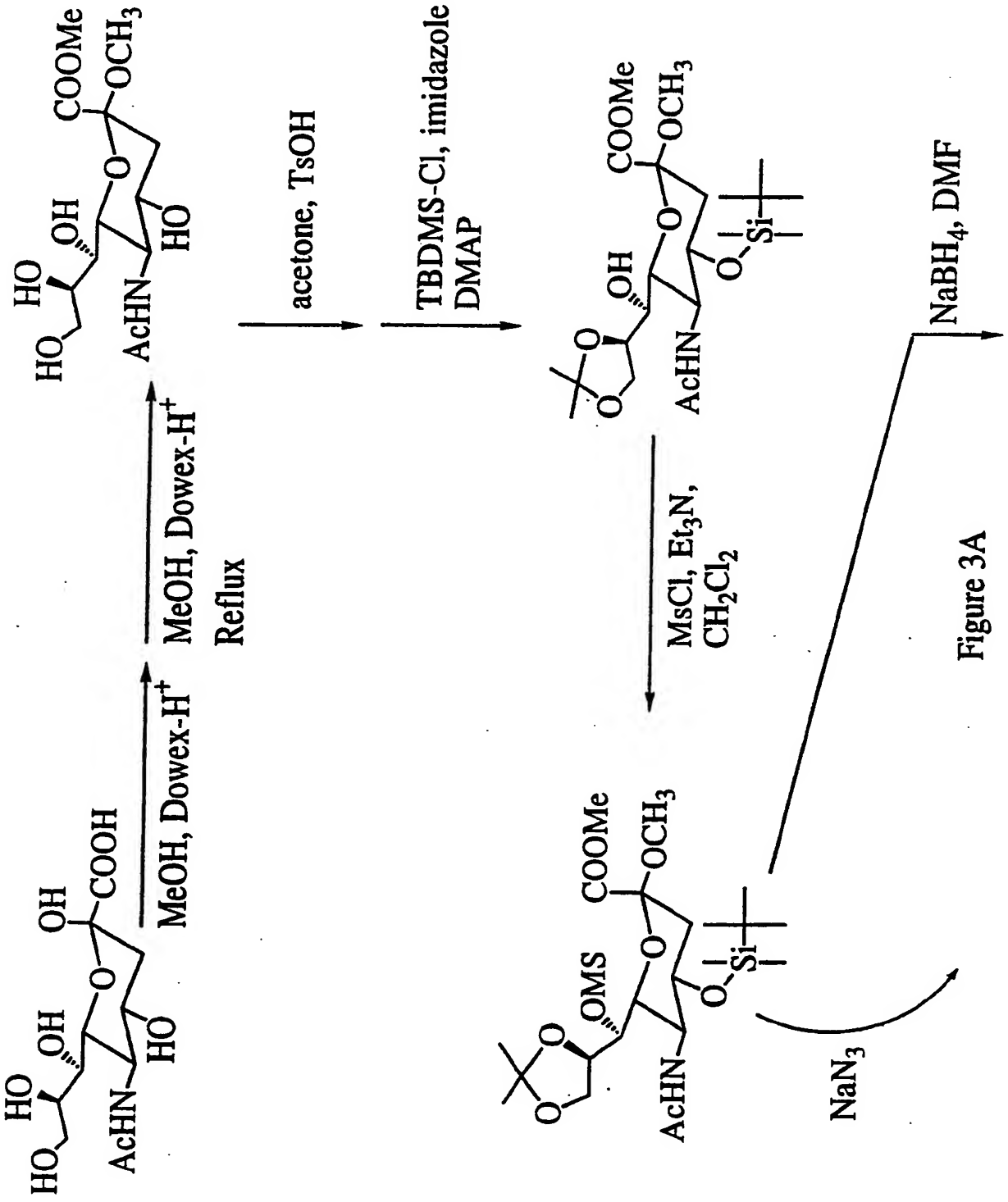


Figure 3A

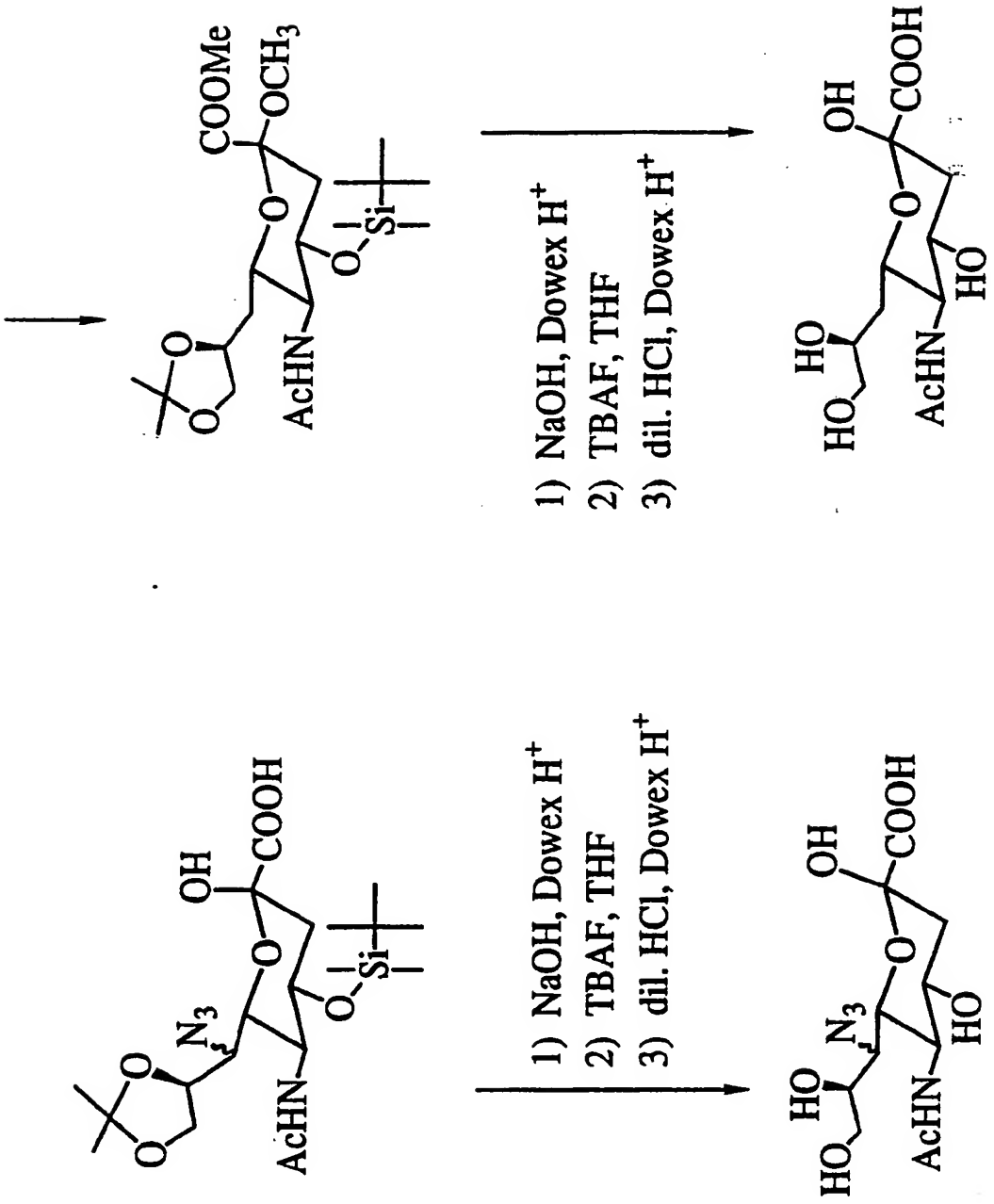

COOMe
OCH3
AcHN
O
Si
1) NaOH, Dowex H+
2) TBAF, THF
3) dil. HCl, Dowex H+
OH
COOH
HO
N3

Figure 3B

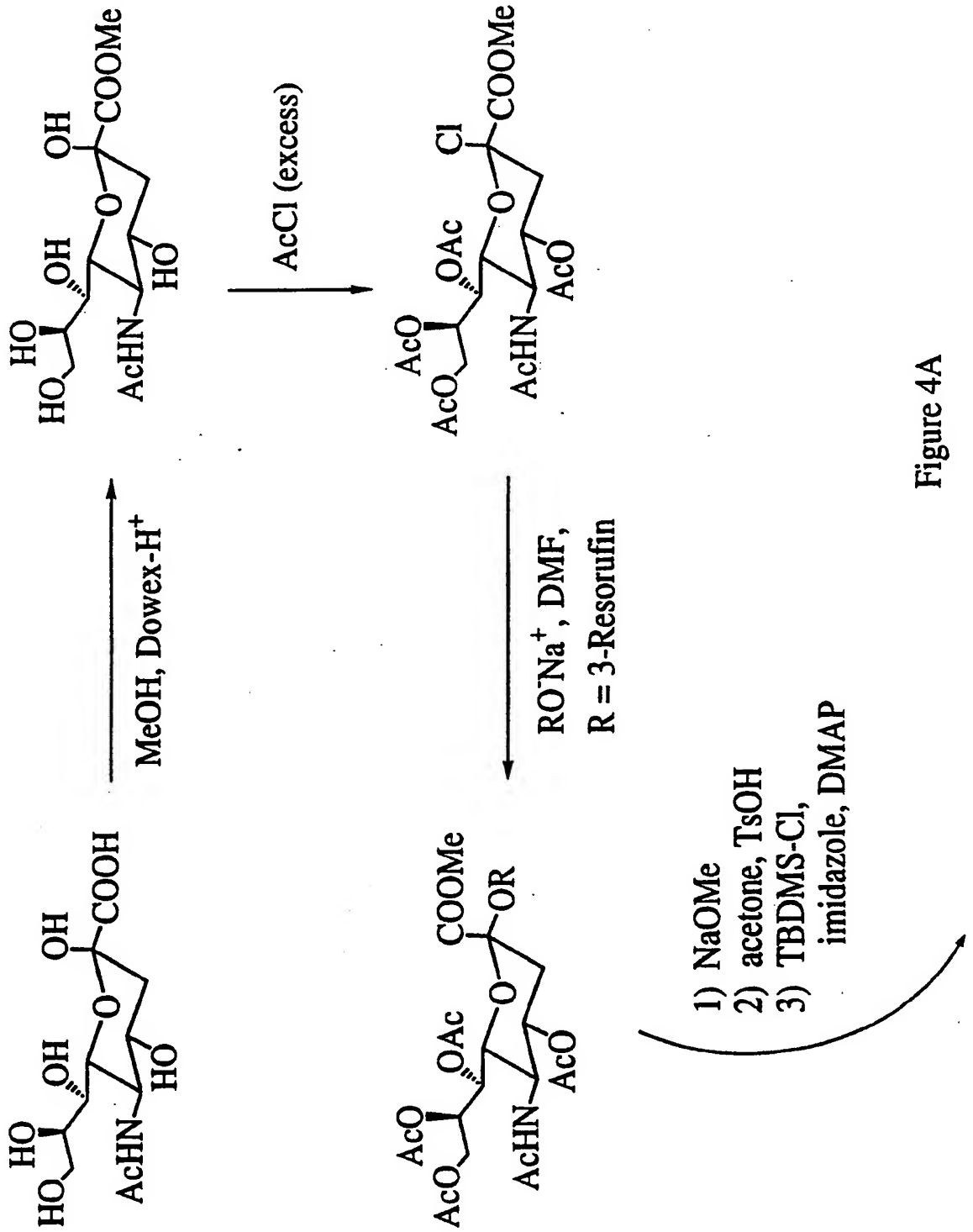
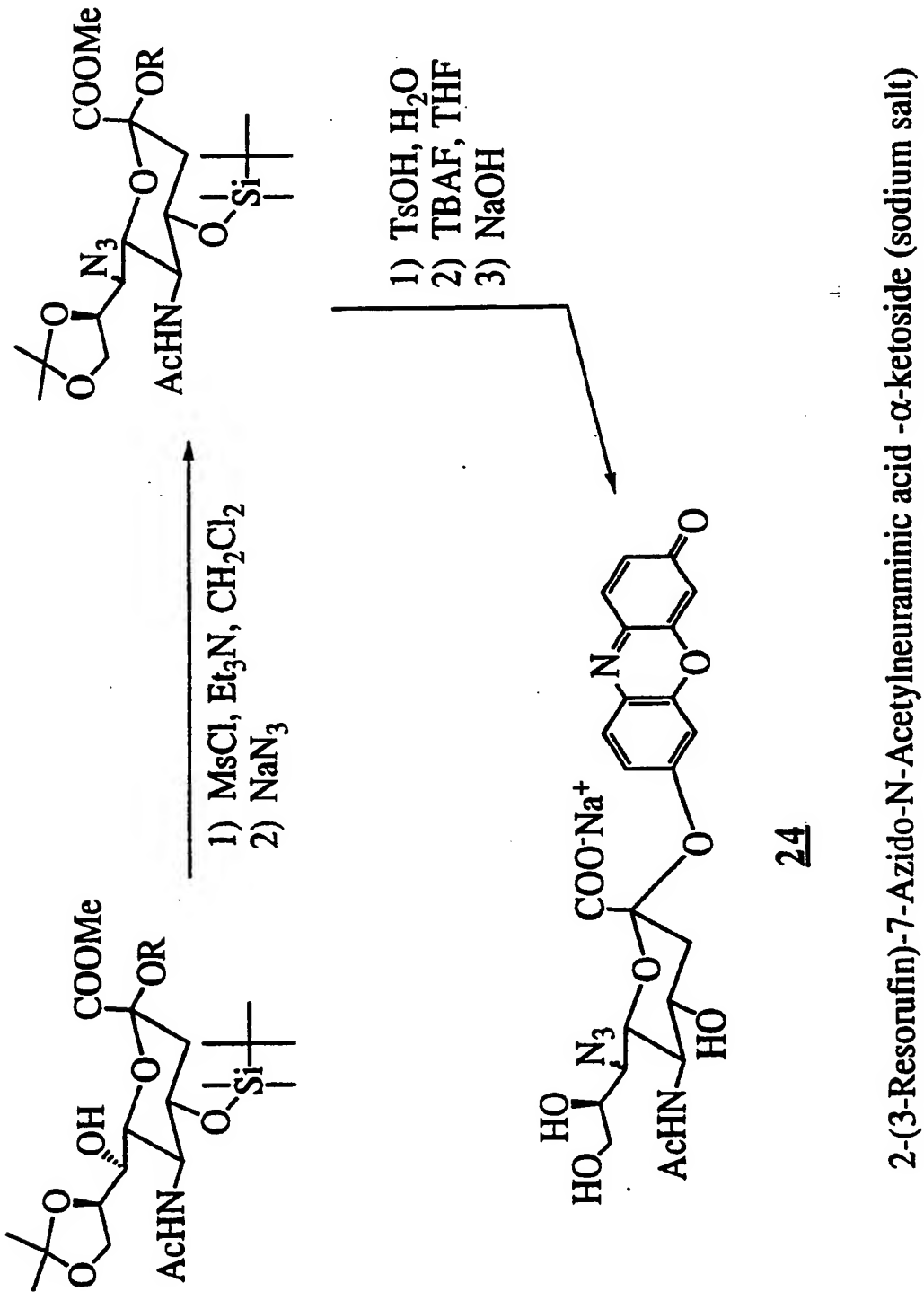


Figure 4A



2-(3-Resorufin)-7-Azido-N-Acetylneuraminic acid -α-ketoside (sodium salt)

Figure 4B

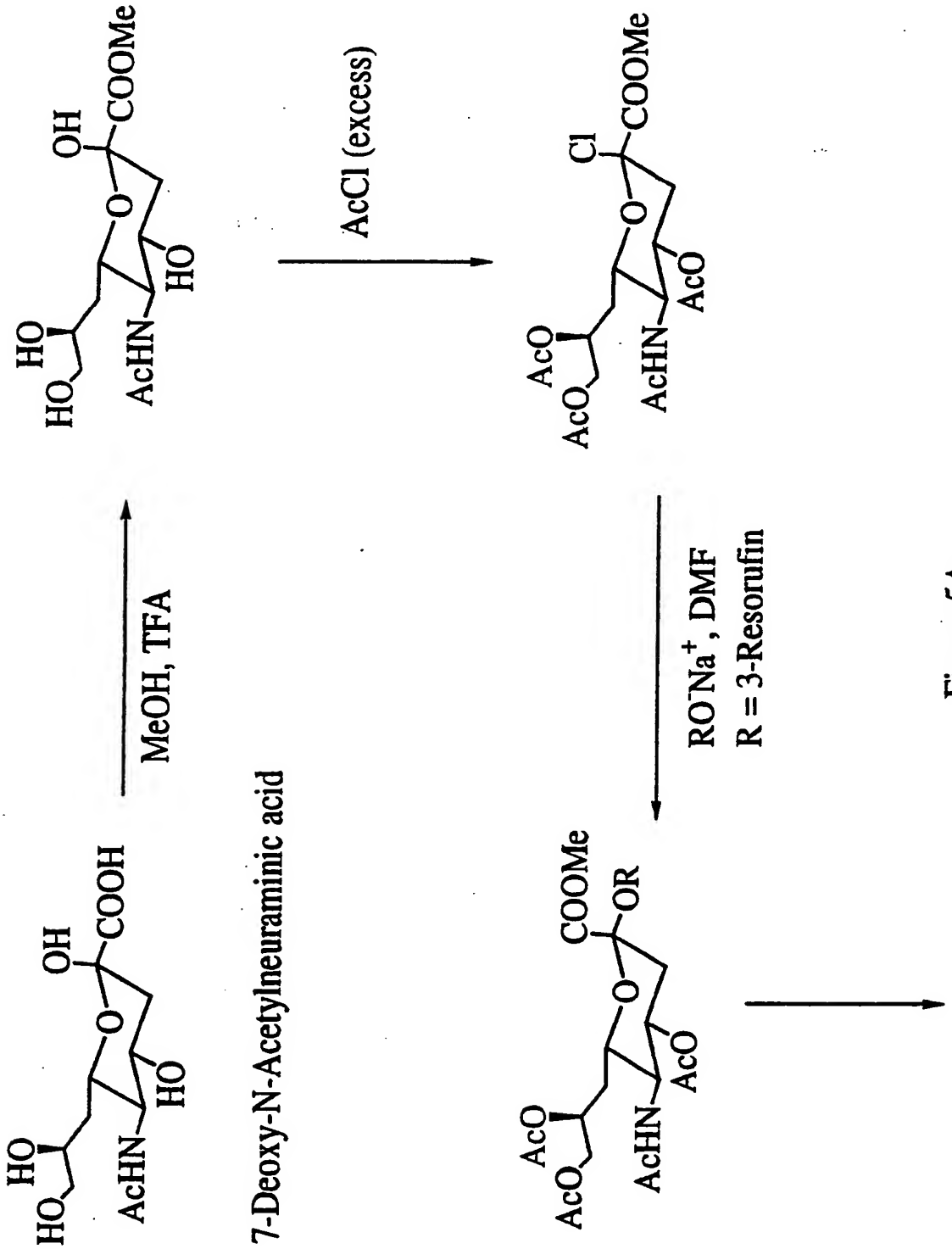
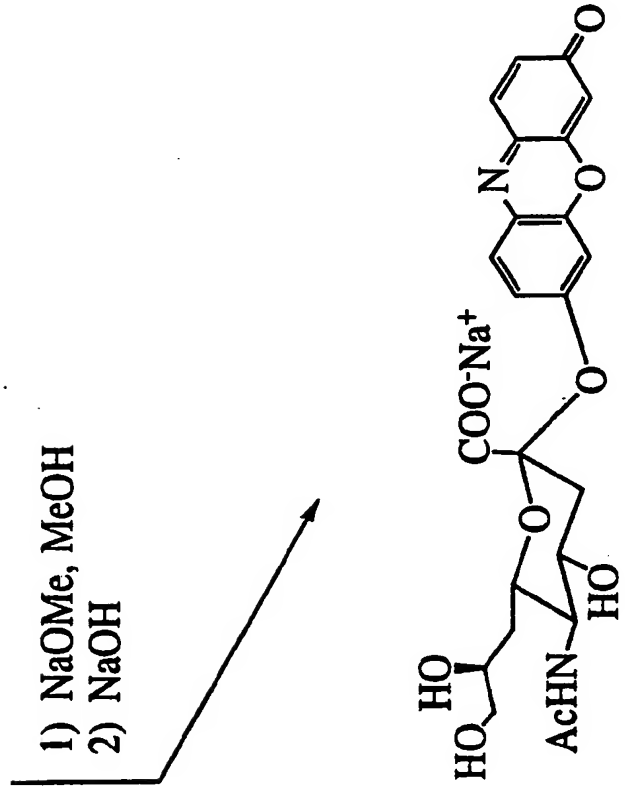

7-Deoxy-N-Acetylneuraminic acid
MeOH, TFA
AcCl (excess)
RO⁻Na⁺, DMF
R = 3-Resorufin

Figure 5A



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2-(3-Resorufin)-7-Deoxy-N-Acetylneuraminic acid -α-ketoside (sodium salt)

Figure 5B

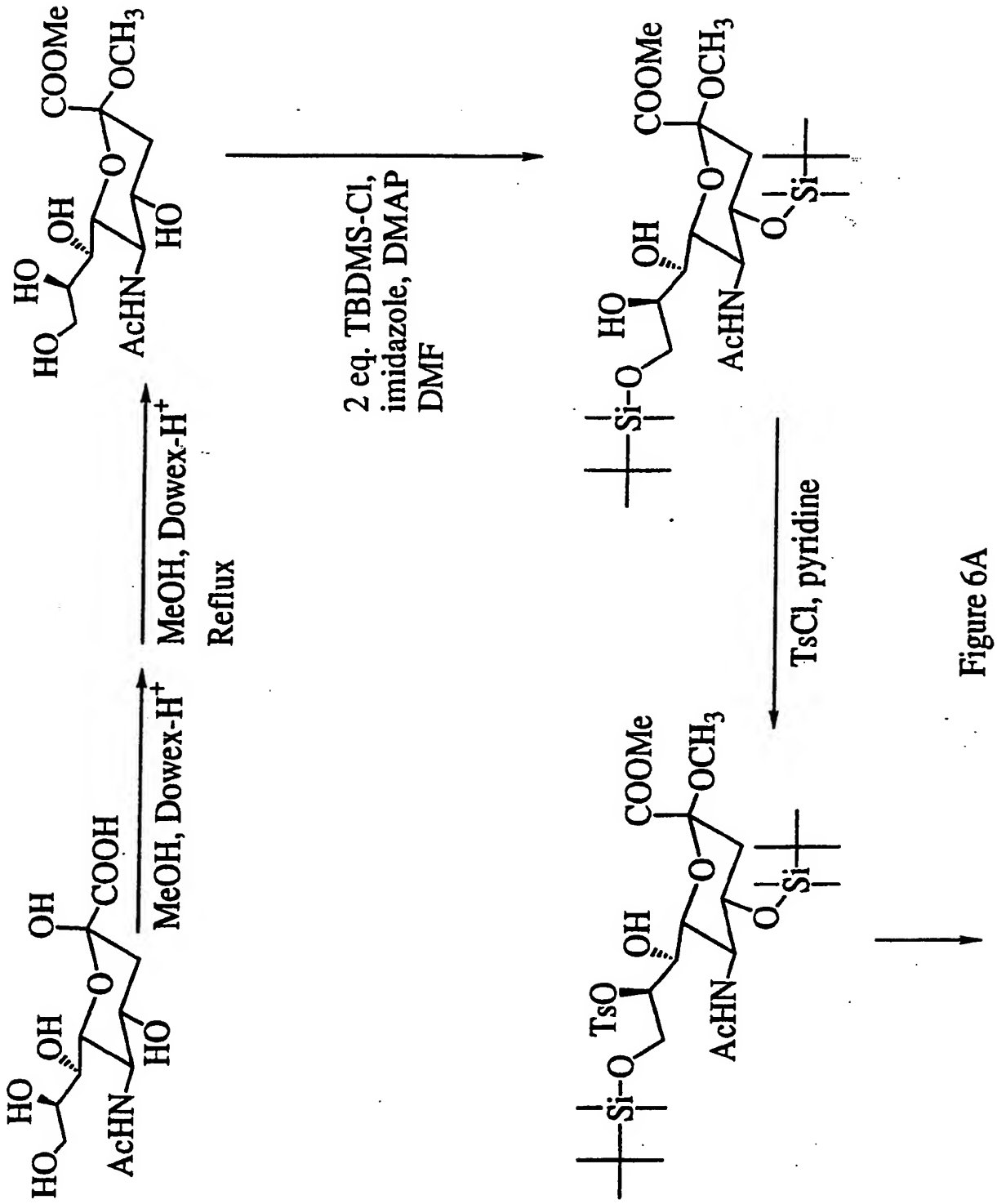

COOH
OH
HO
HO
OH
HO
AcHN
MeOH, Dowex-H+
Reflux
COOMe
OCH3
2 eq. TBDMS-Cl,
imidazole, DMAP
DMF
TsCl, pyridine
TsO
Si

Figure 6A

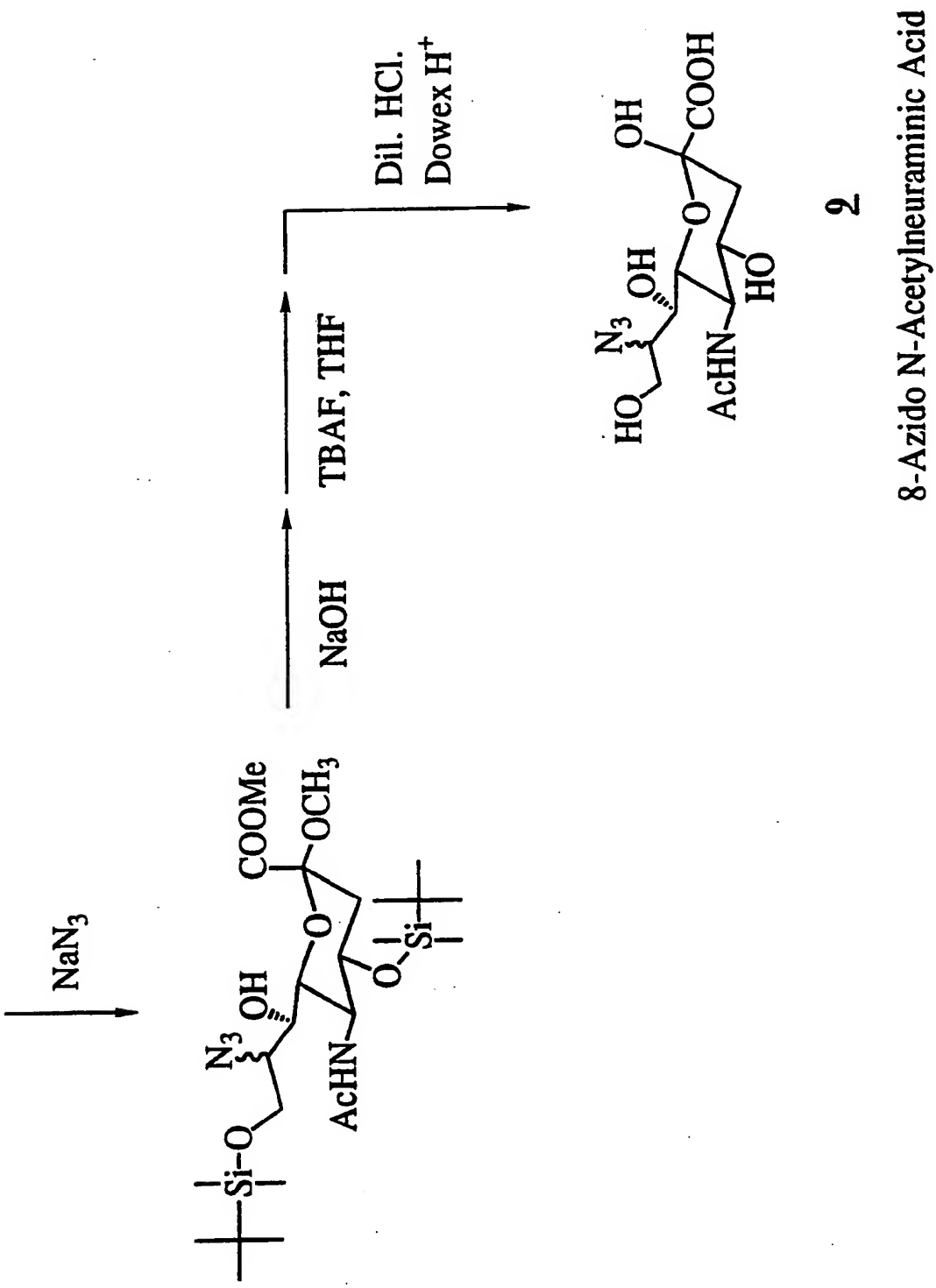


Figure 6B

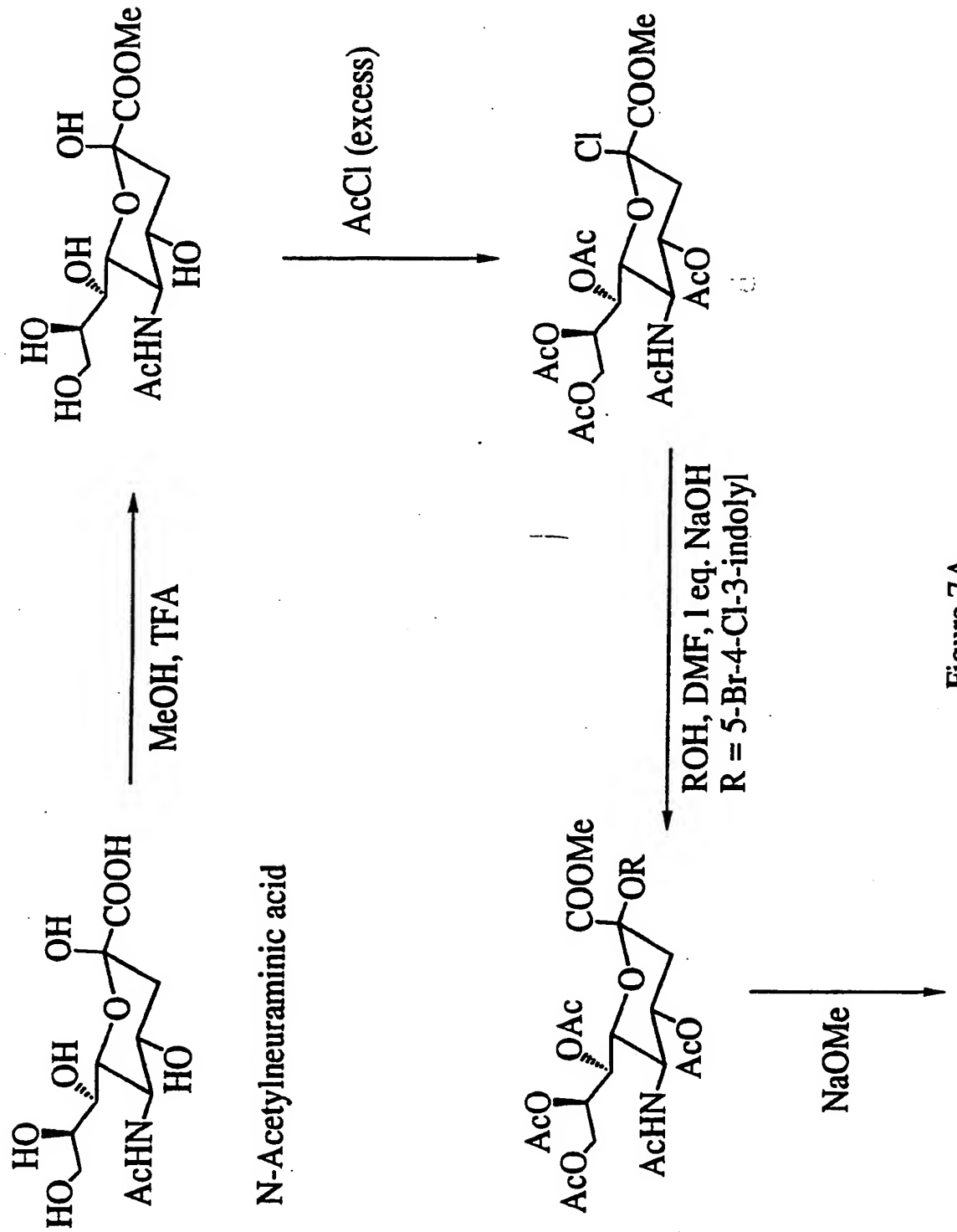


Figure 7A

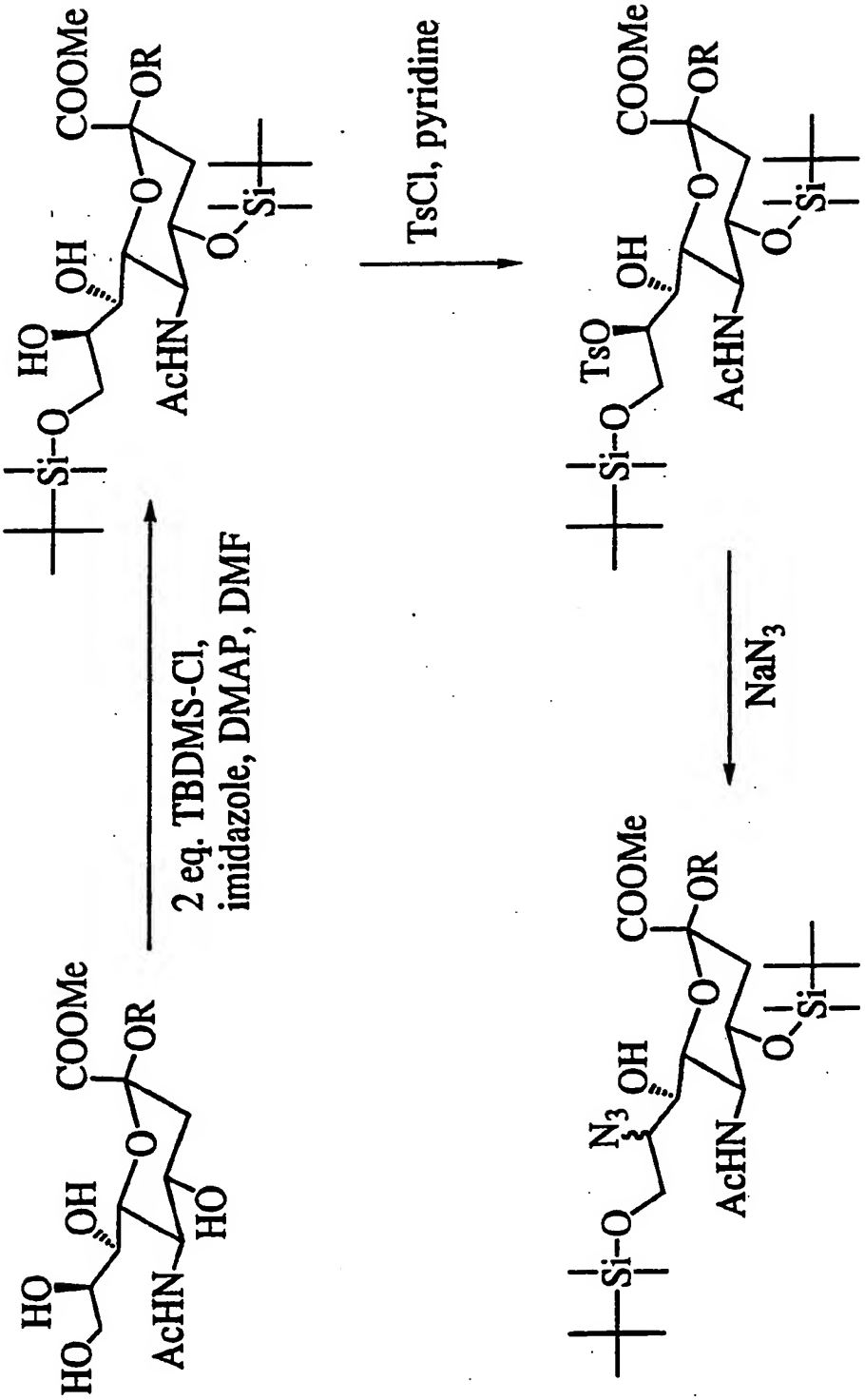
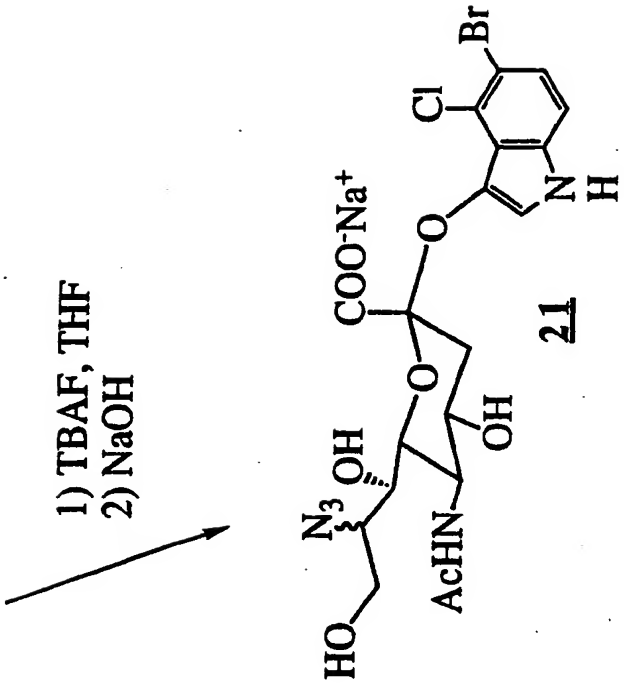


Figure 7B



21

2-{3-(4-Chloro-5-Bromo)-Indolyl}-8-Azido-N-Acetylneuraminic acid
-α-ketoside (sodium salt)

Figure 7C

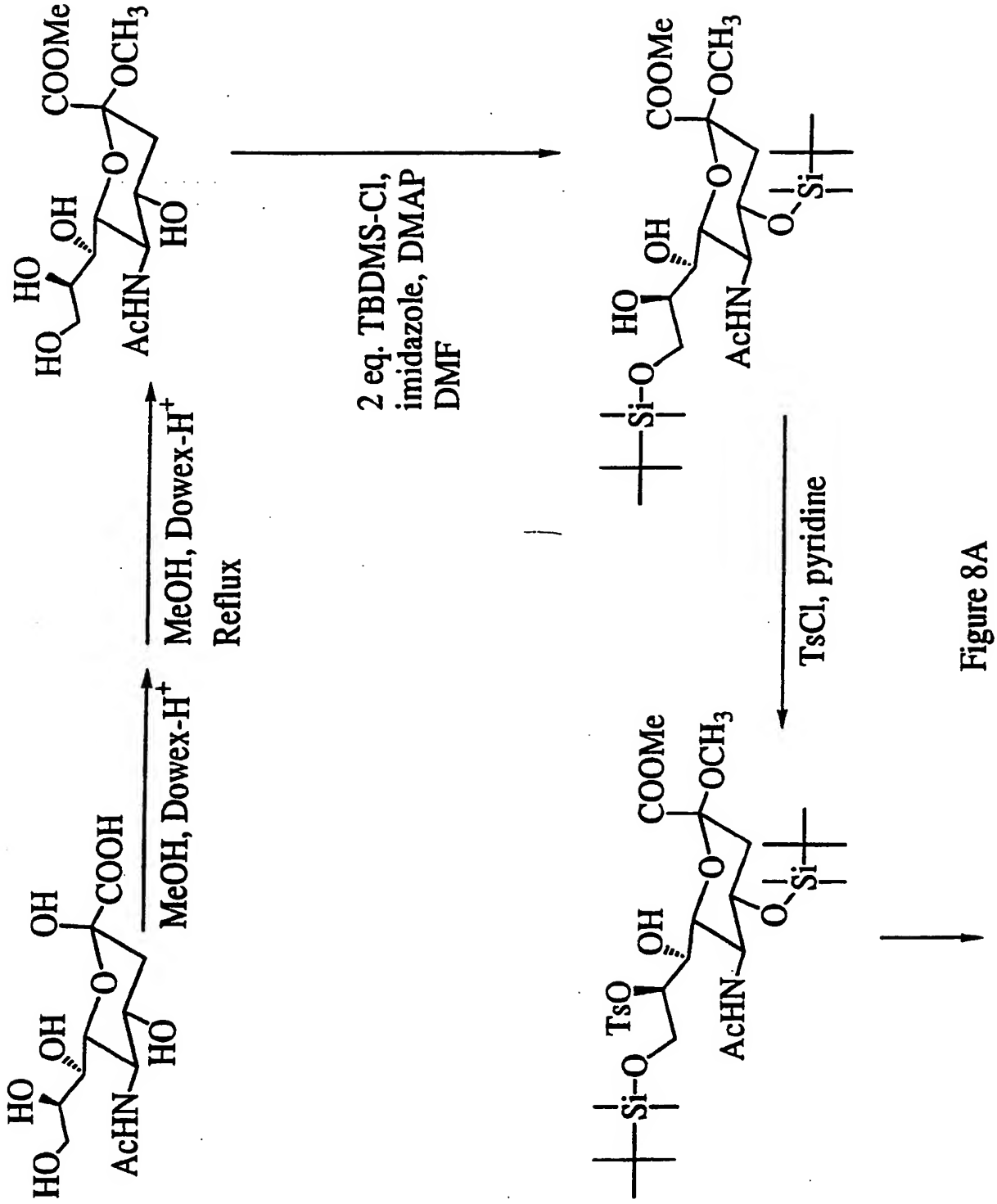
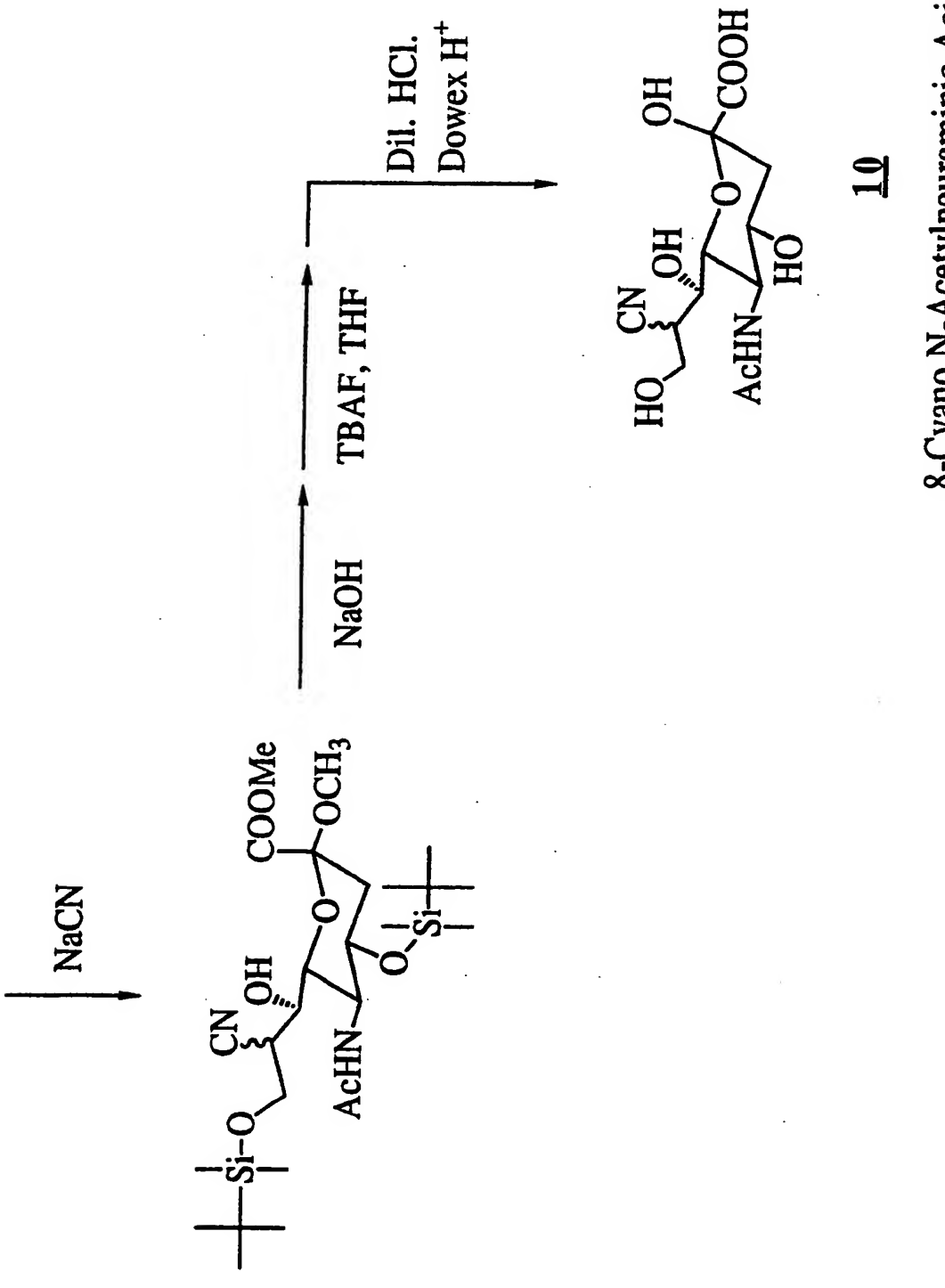


Figure 8A



10

8-Cyano N-Acetylneuraminic Acid

Figure 8B

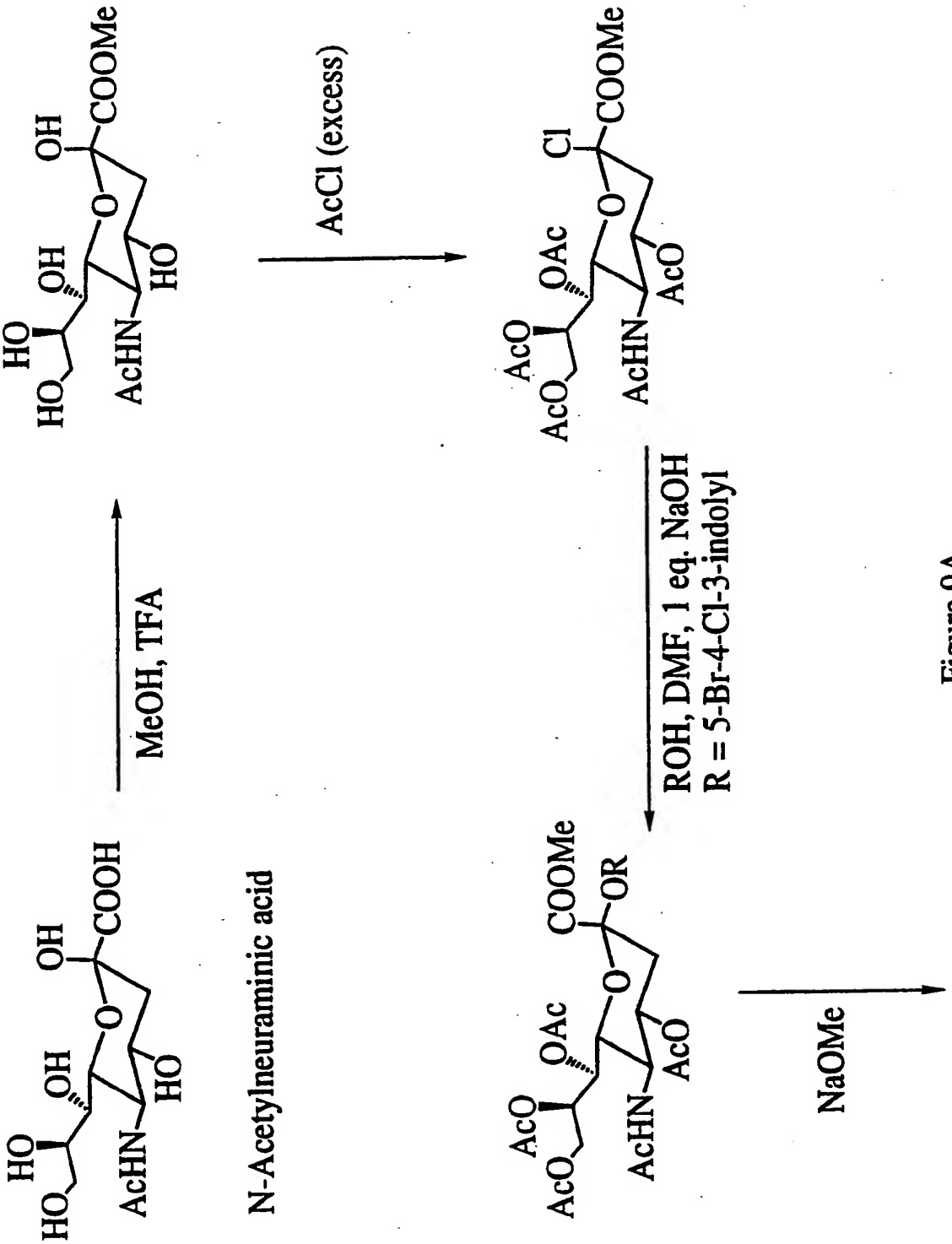


Figure 9A

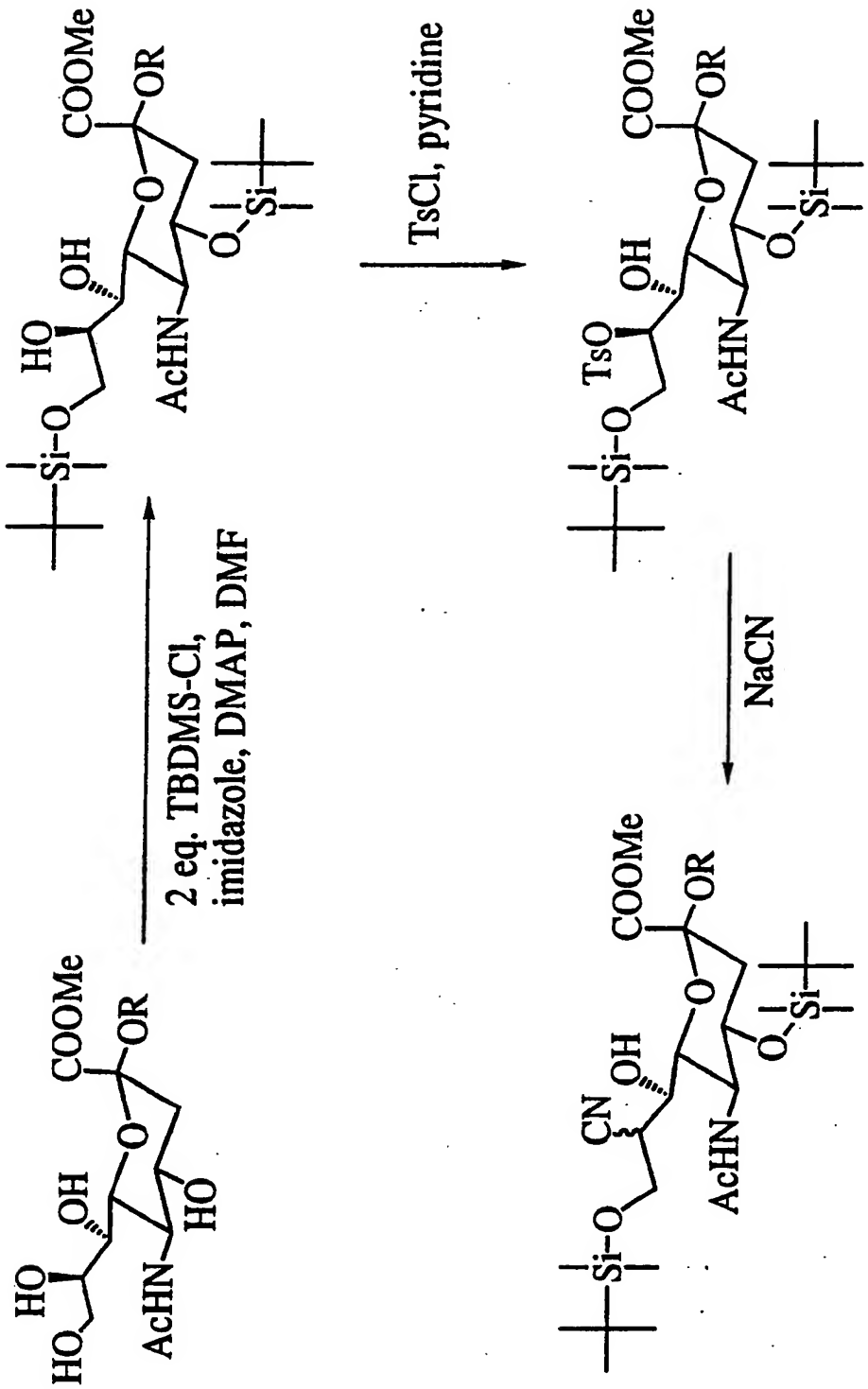


Figure 9B

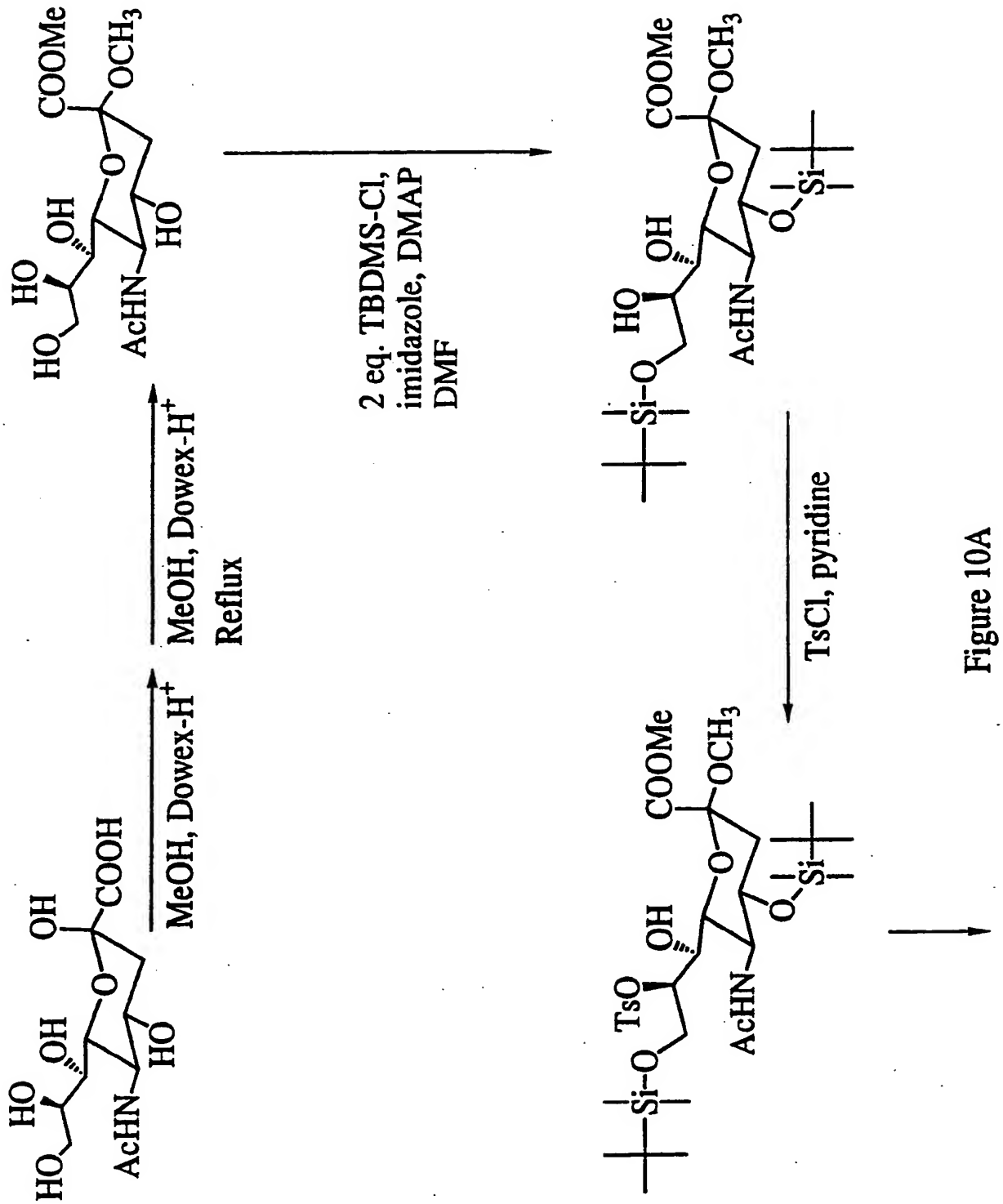


Figure 10A

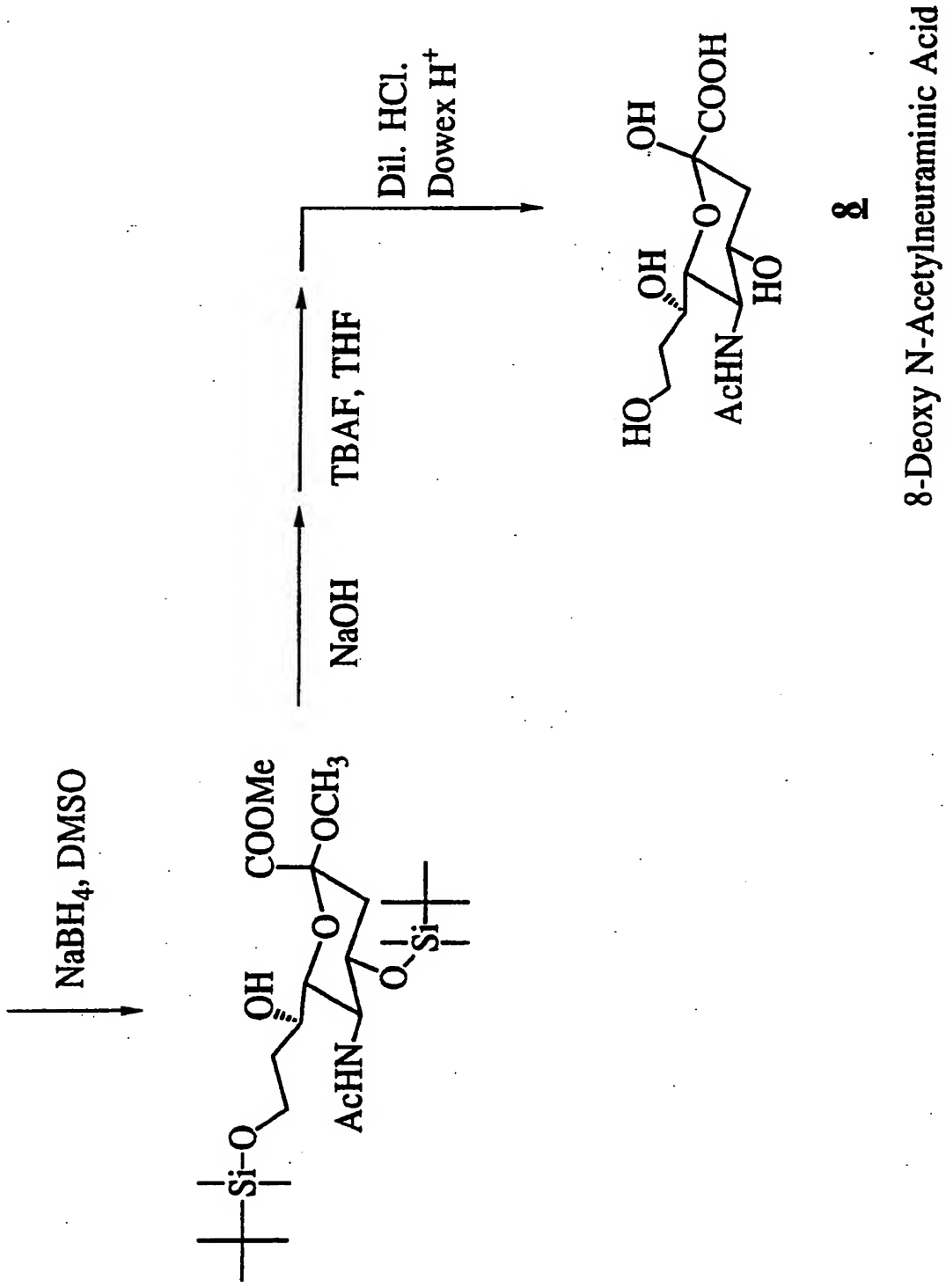


Figure 10B

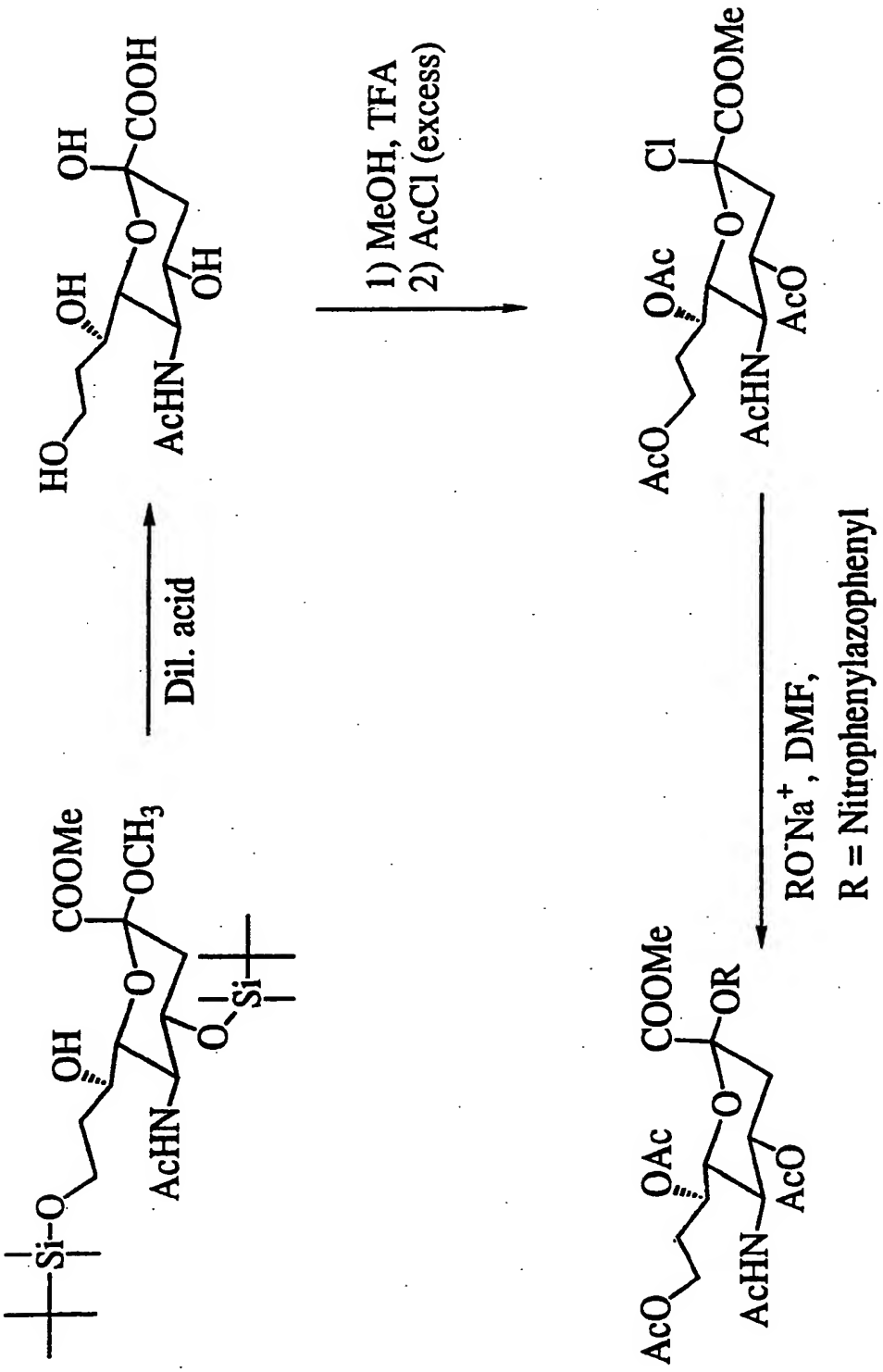
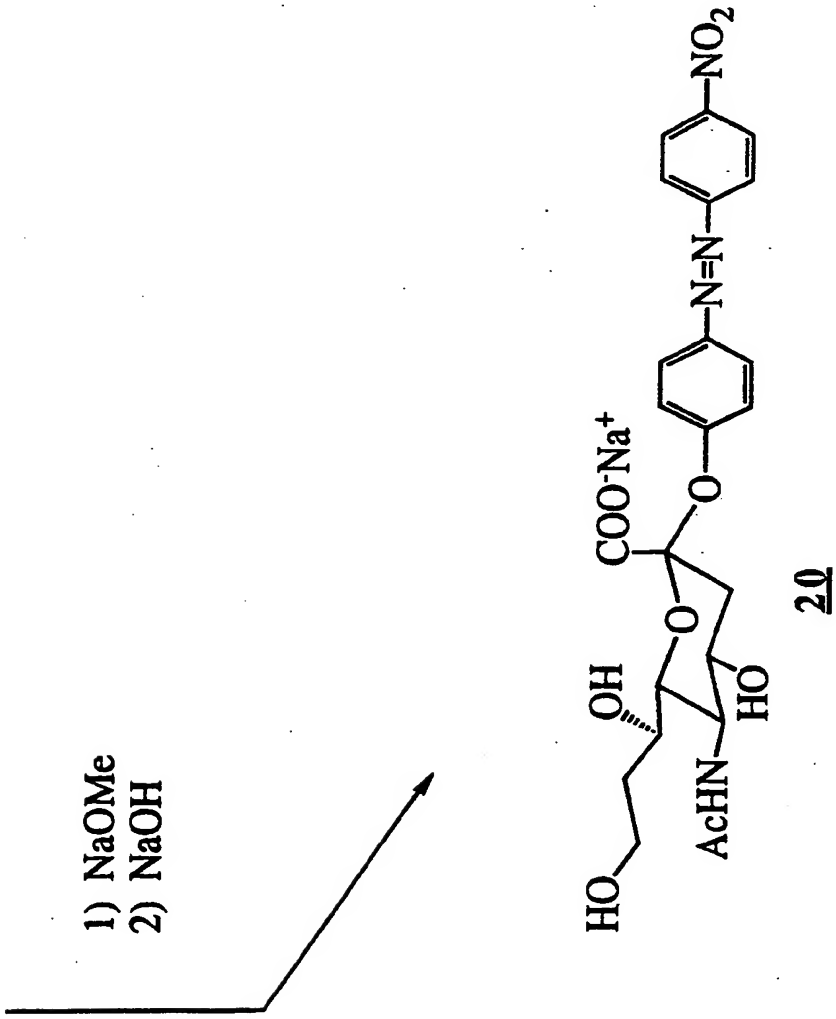


Figure 11A



20

2-{4-(4-Nitrophenylazo)-phenyl}-8-Deoxy-N-Acetylneuraminic acid
-α-ketoside (sodium salt)

Figure 11B

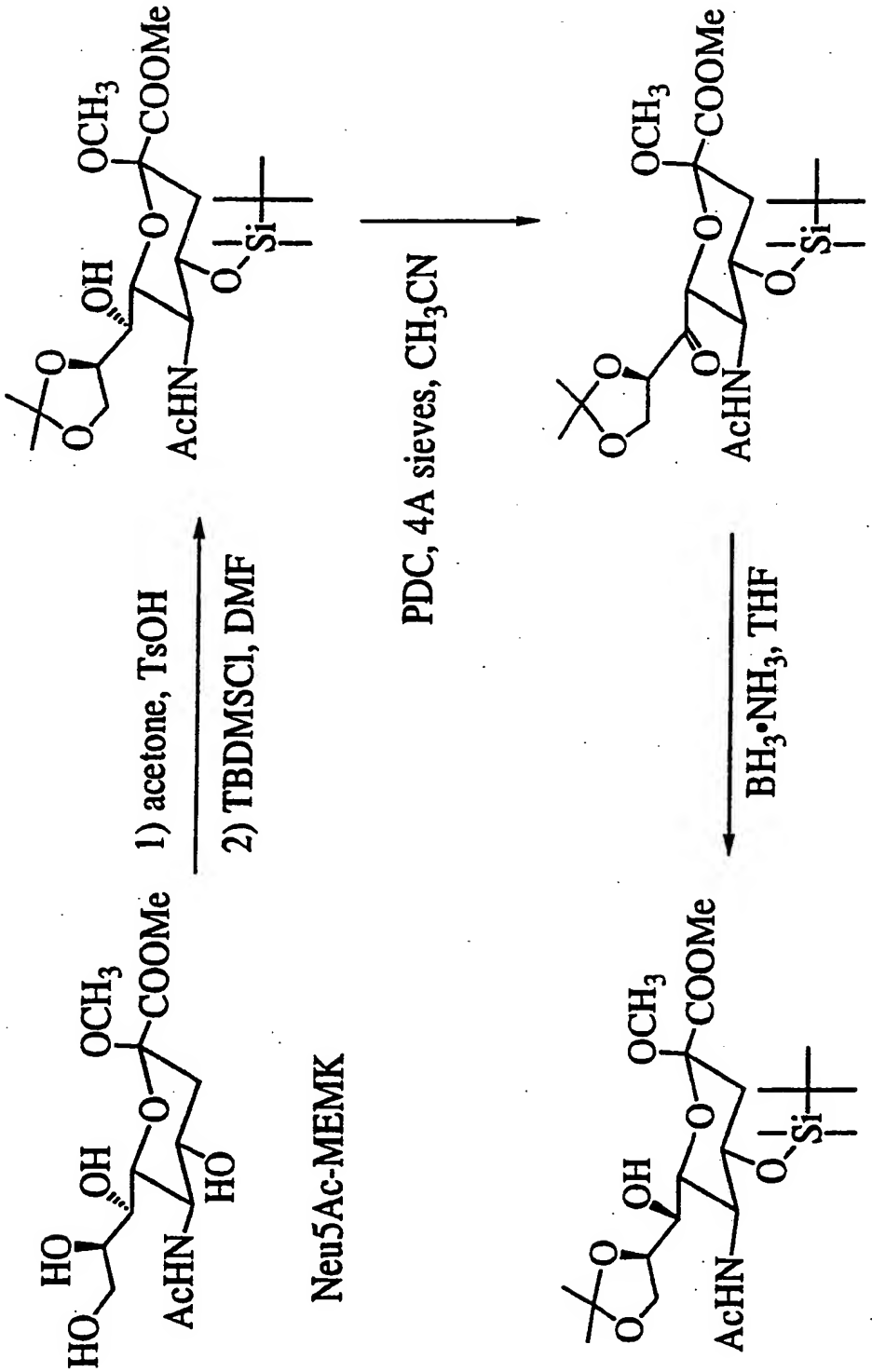


Figure 12A

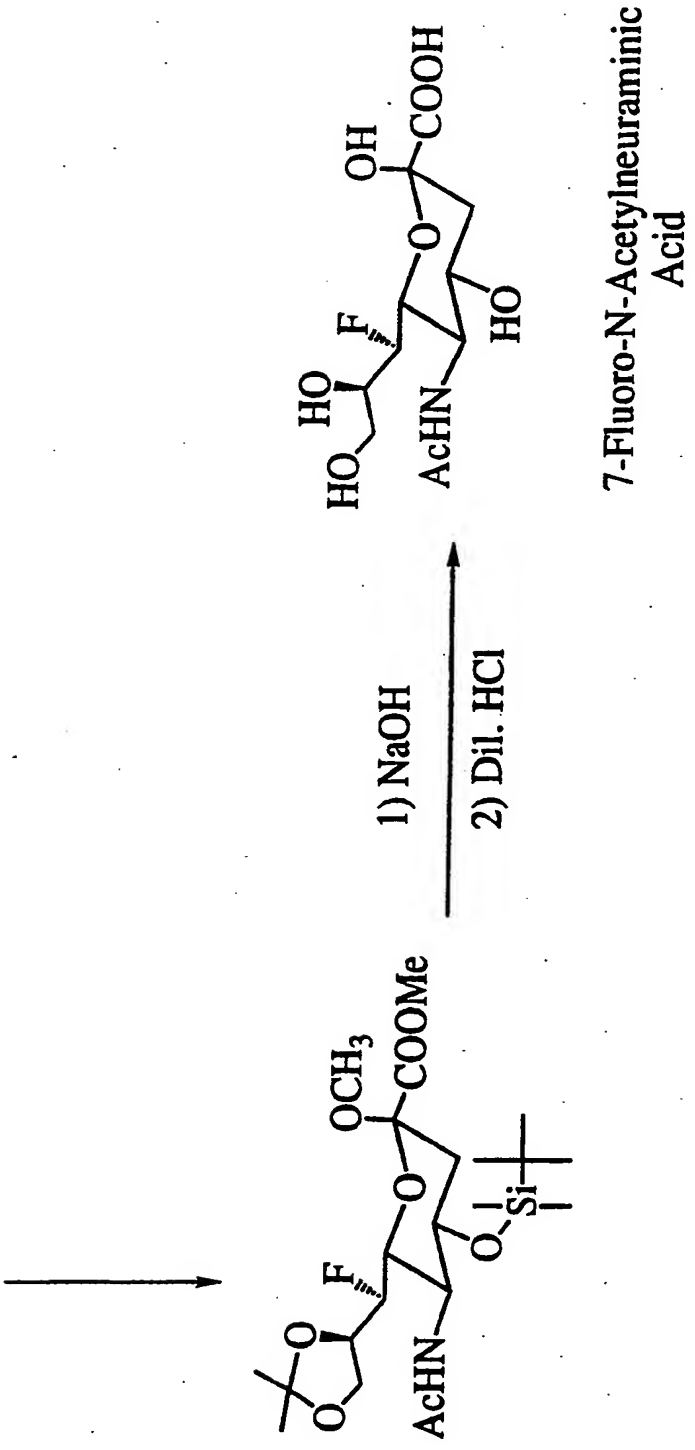


Figure 12B

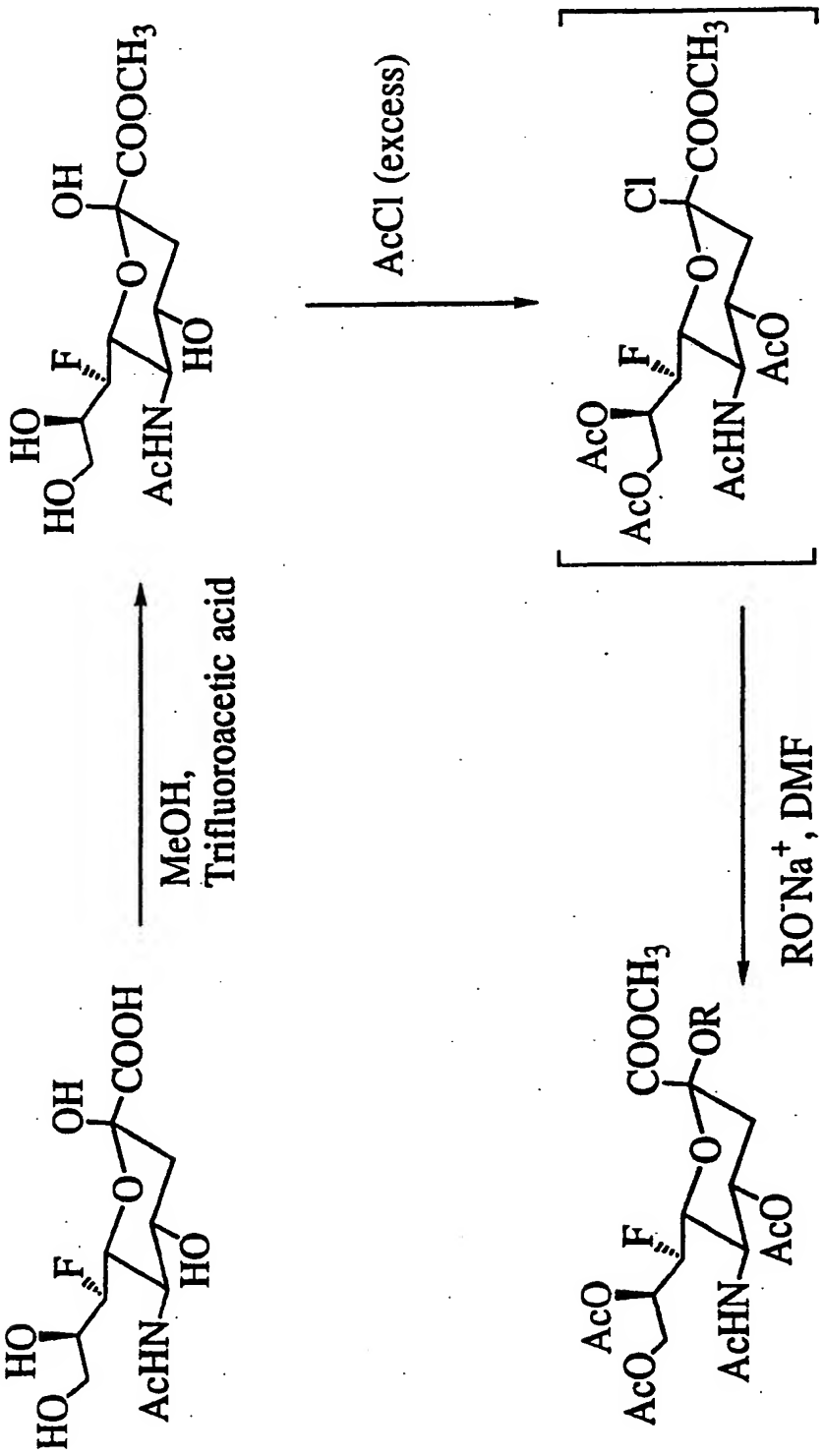
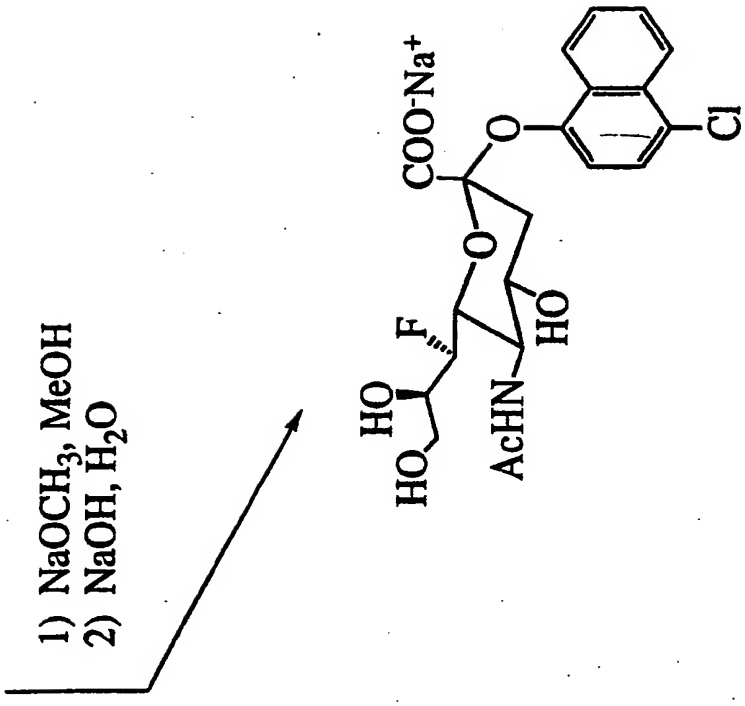


Figure 13A



4-chloro-1-naphthyl-
7-fluoro Neu5Ac (sodium salt)

Figure 13B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/07677

I. CLASSIFICATION SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/70; C12N 1/34; C12N 9/26
U.S. CL: 435/5, 18, 201

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. Cl	435/5,18,201; 534/798 536/17.2,17,3,17.4,17.8,17.9,18.1

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS, CAS, BIOSIS, search terms: Neuraminic, Neuraminidase, Influenza

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US,A 3,950,322 (THOMAS ET AL) 13 April 1976, see entire Document.	1-13 22-30
Y	US,A 4,316,954 (SNOKE ET AL.) 23 FEBRUARY 1982, SEE COLUMN 3,LINES 20-50.	2, 13
A	US,A 4,675,391 (SHIBAYAMA ET AL.) 23 JUNE 1987, SEE ABSTRACT.	3-10 23-30
Y	US,A, 4,731,324 (HUANG ET AL) 15 MARCH 1988, SEE COLUMN 9, LINE 43	3-10 23-30
Y	US,A 4,810,636 (COREY) 07 MARCH 1989, SEE, COLUMNS 1 AND 2.	22-30 1-13
Y	J. KEESEY, "BIOCHEMICA INFORMATION" PUBLISHED 1987 BY Boehringer Mannheim Biochemicals (INDIANAPOLIS, INDIANA), SEE PAGE 176	1-13 22-30

* Special categories of cited documents: [12]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

18 April 1991

09 MAY 1991

International Searching Authority

ISA/US

Timothy Reardon

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☒ Claim numbers 14 -21, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-30, drawn to a method for detecting human influenza Neuraminidase activity and chromogenic substrates used in this method; classified in class 435, subclasses 5,18 and 201.

See attachment.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-30 (Telephone Practice)

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (sheet number 3) (July 1992)

ATTACHMENT A (CONTINUATION OF PART VI)

VI. OBSERVATION WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

II. Claim 31, drawn to a modified N-acetylneuraminic acid, classified in Class 536 subclasses 17.2, 17.3, 17.4, 17.8, 17.9, 18.1.